

AN INVESTIGATION OF THE STRUCTURE OF MANNOSE-CONTAINING
POLYSACCHARIDES

- by -

Robert B. Rashbrook, B.Sc.

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CONTENTS

	<u>Page</u>
<u>INTRODUCTION</u>	1
Galactomannans	2
Glucomannans	4
Mannans	10
The mannans of ivory nut	15

IVORY NUT MANNANS

DISCUSSION

Mannan A	24
Mannan B	37

EXPERIMENTAL

General Methods	38
Extraction of Mannan A	40
Graded acetolysis of Mannan A (1)... ..	41
Deacetylation of acetolysis products	42
Fractionation of oligosaccharide mixture	43
Characterisation of mannobiose	44
Characterisation of mannotriose	51
Graded acetolysis of Mannan A (2)... ..	57
Fractionation of oligosaccharide mixture	58
Separation of disaccharide mixture	60
Attempted characterisation of disaccharides... ..	61

<u>EXPERIMENTAL</u> (Continued)	<u>Page</u>
Separation of trisaccharide mixture... ..	65
Attempted characterisation of trisaccharides ...	65
Investigation of tetraose and pentaose fractions	67
Extraction of Mannan B	69
Graded acetolysis of Mannan B	71
Fractionation of oligosaccharides	72
Examination of the fractions	73
<u>SITKA SPRUCE GLUCOMANNAN</u>	
<u>DISCUSSION</u>	76
<u>EXPERIMENTAL</u> Section (1)	
Attempted purification of the polysaccharide ...	83
Methylation of the glucomannan (1)	85
(2)	90
Section (2)	
Purification of the glucomannan	92
Measurement of percentage hydrolysis ...	93
Periodate oxidation of the glucomannan ...	94
Methylation of the glucomannan (3)	95
Hydrolysis of methylated glucomannan ...	99
Separation and characterisation of hydrolysis products	100
Graded acetolysis of the glucomannan ...	104
<u>SUMMARY</u>	107
<u>BIBLIOGRAPHY</u>	111

INTRODUCTION

Polysaccharides which yield D-mannose as the principal product on hydrolysis are called mannans; in those cases where a second sugar such as glucose or galactose is also produced the mannans are distinguished by the corresponding prefixes e.g. glucomannan, galactomannan etc.

Mannans are found in a wide variety of plants and usually appear to function as reserve polysaccharides. The galactomannans which occur in the ungerminated seeds of many leguminous plants for example, disappear on germination. Many polysaccharides which occur in plants are found closely associated with cellulose in lignified tissues; such carbohydrates are classified as hemicelluloses a term first used by Schultze (1) to describe those alkali-soluble cell-wall constituents which (unlike cellulose) are readily hydrolysed by hot dilute mineral acid. The word is really a misnomer since these substances are not, as Schultze supposed, intermediates in the formation of cellulose. Mainly because of the difficulty of separating the various components, the constitution and properties of the hemicelluloses are still far from being fully understood; there appear however to be three types of molecular species present, namely mannans, galactans, and xylans, some of which contain uronic acid residues. The relationship between

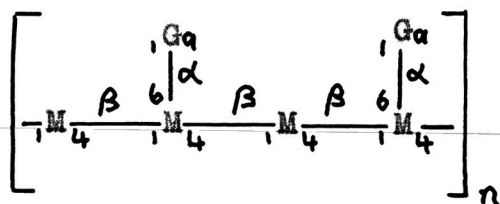
these polysaccharides and cellulose on the one hand, and lignin on the other is still obscure; it is impossible to decide between the alternatives of a very close physical association and some kind of chemical link. Reserve carbohydrates, such as the mannans of ivory nut Phytelephas macrocarpa, which are stored in the form of greatly thickened cell-walls, are also classed as hemi-cellulose, since they closely resemble the polysaccharides of lignified tissues.

Mannans have also been isolated from a number of other sources, notably yeast, the red seaweed Porphyra umbilicalis, and from the products of Penicillium Charlesii G. Smith grown on glucose solution.

GALACTOMANNANS

As Wise and Appling (2) and Anderson (3) have shown, galactomannans are common constituents of the ungerminated seeds of many leguminous plants. They occur as mucilages in the endosperm of the seeds from which they can be extracted with water. The galactomannan content varies from species to species but may be as high as 40%. Structural investigations of a number of these polysaccharides have been made and certain striking similarities between them noted. All contain more mannose than galactose with the

exception of a galactomannan from lucerne seed studied by Hirst, Jones, and Walder (4) in which the ratio of galactose to mannose is 2:1. Hydrolysis of the methylated galactomannans from fenugreek seed (5,6), carob seed (7,8), guar seed (9,10), the Kentucky coffee bean (11), and lucerne and clover seed (12), in all cases gave 2:3:4:6 tetramethyl D-galactose, 2:3:6 trimethyl D-mannose, and 2:3 dimethyl D-mannose. These results show clearly that the galactose occupies a terminal position in the molecule and is attached to a backbone of mannose residues by 1:4 and/or 1:6 linkages. Further evidence for the structure of guaran, the polysaccharide from guar seed, has been provided by Whistler and his co-workers (13,14,15,16). From the partial hydrolysis products of the galactomannan they have isolated the disaccharides 4-O- β -D-mannopyranosyl- β -D-mannopyranose and 6-O- α -D-galactopyranosyl- β -D-mannopyranose, and the trisaccharides O- β -D-mannopyranosyl-(1 \rightarrow 4)-O- β -D-mannopyranosyl-(1 \rightarrow 4)- β -D-mannopyranose and O- α -D-galactopyranosyl-(1 \rightarrow 6)-O- β -D-mannopyranosyl-(1 \rightarrow 4)- β -D-mannopyranose. These results have led them to postulate the following structure for guaran:



There can be little doubt that this formula closely represents the structure of the polysaccharide molecule, and it seems highly probable that all these galactomannans have a similar constitution, that is a chain of β -1:4-linked mannose residues to which galactose residues are attached at intervals by α -1:6-linkages. Hirst and Jones in their paper on the galactomannan of carob gum (7) have noted that other workers (17,2,18) on the same polysaccharide report different values for the ratio of galactose to mannose, and suggest that galactose may be stored in the plant by being attached randomwise to a mannan chain. This view is consistent with the later work quoted above; the alternative possibility that the polysaccharides are mixtures appears unlikely, since Hirst and Jones were unable to achieve any fractionation of the methylated derivative into components of different physical properties.

GLUCOMANNANS

Glucomannans appear to be more widely distributed in nature than galactomannans but they have been less intensively studied. This may be partly due to the fact that elucidation of the structure of these polysaccharides presents considerable difficulty. As a rule glucomannans are not readily purified and the homogeneity of the products obtained is often doubtful.

Again they are frequently insoluble or only slightly soluble in water and are not readily hydrolysed, methylated or acetylated. The close similarity in properties between the methylated derivatives of mannose and glucose is a further complicating factor in structural determinations.

Konjak Mannan (19,20)

Nishida and his co-workers extracted a glucomannan from the corms of Conophallus konjak by means of superheated water. Hydrolysis of the polysaccharide yielded mannose and glucose in the ratio 2:1. Methylation and hydrolysis gave 2:3:6 and 2:3:4-tri-O-methyl-D-mannose, so that both 1:4 and 1:6 linkages appear to be present. A number of oligosaccharides were isolated by partial acetolysis followed by deacetylation of the products, including a mannobiose, a glucomannobiose, and a trisaccharide containing one glucose and two mannose units.

Torigata (21) has reported more recently that glucuronic acid is also present in the molecule.

Iles Mannan (22,23)

Iles mannan meal is prepared from the tubers of the Amorphophallus oncophyllus and A. variabilis plants which are found in Indonesia. It contains a glucomannan and a smaller amount of a glucan, as well as cellulose, lignin, etc.

The mannose content is variable (43% - 75%).

Rebers and Smith separated the glucomannan and the glucan by methylation followed by fractional precipitation from a number of solvents. Hydrolysis of the purified glucomannan gave a mixture of 2:3:6-tri-O-methyl-D-glucose and 2:3:6-tri-O-methyl-D-mannose which were separated by selective furanoside formation. The low optical rotation (-41° in ethanol) of the methylated polysaccharide suggested that it is predominantly β -linked. Partial acetolysis of the crude "mannan" followed by deacetylation and separation of the products on a cellulose column led to the isolation of three crystalline disaccharides: 4-O- β -D-glucopyranosyl- α -D-mannopyranose, cellobiose, and 4-O- β -D-mannopyranosyl- α -D-glucopyranose. Thus the glucomannan appears to consist of straight chains of β 1:4-linked mannose and glucose units.

The Glucomannans of Iris seed (24)

Glucomannans have been extracted with dilute (10%) alkali from the seed of Iris ochroleuca and I. sibirica. The two polysaccharides were very similar in composition and properties. Both had low specific rotations suggesting predominantly β -links in the molecule. On hydrolysis they gave D-mannose and D-glucose in the ratio 1:1 (approximately)

(97%), and D-galactose (3%). Methylation and hydrolysis revealed that the galactose was present as end-group, a little mannose and glucose end-group also being detected. The trimethyl fraction consisted of a mixture of 2:3:6-tri-O-methyl-D-glucose and 2:3:6-tri-O-methyl-D-mannose.

The Glucomannans of Lily bulbs

Recently Andrews, Hough and Jones (25), who have been conducting an extended investigation of mixed mannans, examined the glucomannans which can be extracted with water from the bulbs of certain Lilies. The polysaccharides studied were those from Lilium umbellatum, L.henryii and L.candidum. All had a mannose to glucose ratio of about 2:1 and low specific rotations. The glucomannans of L.umbellatum and L.henryii have been methylated and hydrolysed, the main products in each case being 2:3:6-tri-O-methyl-D-glucose and 2:3:6-tri-O-methyl-D-mannose. The end-group consisted entirely of 2:3:4:6-tetra-O-methyl-D-glucose. Thus it may be inferred that the glucomannans are composed of β -1:4-linked mannose and glucose residues, terminated at the non-reducing end by a glucose residue. The L.umbellatum polysaccharide is branched at a glucose unit since 2:4-di-O-methyl-D-glucose has been identified in the hydrolysate. It is likely that the L.henryii glucomannan is

also branched at one or two points per molecule.

Wood Mannans

"Mannans" can be isolated in an impure state from wood by alkaline extraction, following a pre-treatment with a delignifying reagent such as chlorine dioxide. Remarkably little structural work has been carried out on wood mannans so that classification is still uncertain, however they appear to fall into the category of glucomannans. Until recently the emphasis has lain rather on the estimation of the total mannan content of various woods rather than on the isolation of the polysaccharides themselves. In this connection a significant difference in the composition of the wood of conifers (Gymnospernae) and hardwoods (Angiospermae) has been noted. Mannans have been detected in a large number of softwoods (26) sometimes in considerable quantity (up to 10%). In hardwoods on the other hand, mannan may be entirely absent or occur only to the extent of about 1%. (27,28).

Hemicellulose, and particularly the mannan fraction of hemicellulose is of considerable technical importance in the paper and wood-pulp industry. In general it may be said that the presence of hemicellulose exerts a beneficial influence in paper-making in improving the tensile strength etc. of the product. In the viscose rayon industry however

the low solubility of the hemicellulose derivatives leads to clogging of the spinnerets, necessitating a wasteful pre-treatment of the pulp with hot alkali to remove the unwanted constituents.

Structural studies on wood mannans can be very briefly summarised. Leech (29) has acetolysed " α - cellulose" from Slash Pine and after saponification of the products has shown that cellobiose, a mannobiose chromatographically identical with that obtained from ivory nut mannan, and a glucosyl-mannose are all present in the mixture.

Jones and his co-workers (30) have recently isolated a glucomannan from the extractive-free sawdust of Loblolly Pine. The polysaccharide gave D-mannose, D-glucose and a little D-galactose on hydrolysis. On methylation and hydrolysis the galactose appeared entirely as end-group, the main products being 2;3;6-tri-O-methyl-D-glucose and 2;3;6-tri-O-methyl-D-mannose. By partial acid hydrolysis of the sawdust a disaccharide was obtained which was identified as 4-O- β -D-glucopyranosyl-D-mannopyranose. The polysaccharide thus appears to have a basically similar structure to the glucomannans already considered.

Mixed Mannans from Reindeer Moss

Aspinall, Hirst and Warburton (31) have obtained two

polysaccharides by extracting Reindeer Moss, Cladonia alpestris, with dilute and concentrated alkali. Both gave D-galactose, D-glucose and D-mannose on hydrolysis. Methylation and periodate oxidation studies indicate that these polysaccharides are mixtures of complex, highly-branched molecules in which mannose and glucose residues form the backbones and the galactose residues are present entirely as end-groups. It is not possible to say whether the galactose is associated with the mannose or the glucose or both.

MANNANS

The true mannans show a greater diversity of structural types than the mixed mannans which have been considered so far. It is noticeable however that the β -1:4 link which is such a characteristic structural feature of the latter types of polysaccharide is also the predominant linkage in mannans obtained from the higher plants.

Salep Mannan

The extraction of orchid tubers (Orchis sp) with cold water yields a mannan which can be precipitated with alcohol. This mannan has been studied by several workers (32-36) and application of the classical methylation technique

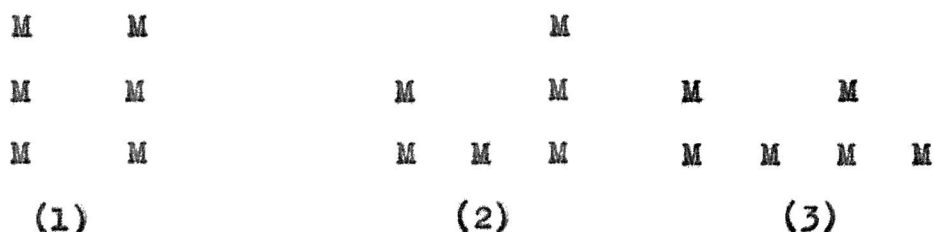
has shown that the polysaccharide is 1;4-linked and that it has a chain length of 70-80. Husemann (36) has postulated that the water-solubility of this mannan, in contrast to those of similar structure, is due to the presence of acetyl groups on some of the mannose residues.

Yeast Mannan

Yeast mannan was first obtained by Schutzenberger (37) who isolated it by extraction with hot water. Aqueous extraction has also been employed by other workers (38,39,40), but in more recent investigations (41,42,43) extraction with hot dilute alkali has been preferred. As might be expected, the polysaccharides obtained by different workers differ considerably in properties and in optical rotation. The high positive values quoted for the latter suggest that

α -links predominate in the molecule. Haworth, Hirst, and Isherwood (42) obtained a fully methylated derivative which on hydrolysis gave 2:3:4:6-tetra-O-methyl-D-mannose, 2:3:4-tri-O-methyl-D-mannose, and 3:4-di-O-methyl-D-mannose. Later, Haworth, Heath, and Peat (44) confirmed that tetra-, tri-, and dimethyl mannose were produced in equimolecular proportions and that the structures assigned to the tetra- and dimethyl sugars were correct. The trimethyl fraction however, was shown to consist mainly of 2:4:6- and 3:4:6-

tri-O-methyl-D-mannose in equimolecular proportions, 2:3:4-tri-O-methyl-D-mannose being present only to the extent of about 10%. They proposed that one of the three following formulae represented the repeating-unit in the molecule:-



Linsteadt (45) obtained results in agreement with the above structures by periodate oxidation, hydrolysis, and tritylation studies on the polysaccharide.

Recently Smith and his co-workers (71) have shown that the polysaccharide associated with yeast invertase is identical with the yeast mannan described above and is not a 1:3-linked polysaccharide as reported by Fischer and Kohtes (72).

Mannocarolose

The mould Penicillium Charlesii G. Smith, produces a polysaccharide mixture when grown on glucose solutions. This can be separated into two components by fractional precipitation with alcohol, one a galactan, and the other a mannan which has been named mannocarolose. This polysaccharide

while mannitol occurs in large quantities in the brown seaweeds.

Jones (48) has isolated a mannan from the red seaweed, Porphyra umbilicalis, by extraction with hot 20% sodium hydroxide. Methylation and periodate oxidation studies showed that the polysaccharide was branched, with an average of one branch-point per twelve β -1:4-linked mannose residues.

Fungal and Bacterial Mannans

Mannans have been found in certain fungi (49) and in several species of bacteria (50,51). Little structural work has been done on these polysaccharides though Rydon and his associates (52) have methylated a mannan from Bacillus anthracis and shown that 2:3:6-tri-O-methyl D-mannose was produced on hydrolysis.

ENZYMIC DEGRADATION OF MANNANS

The use of enzymes to determine the fine structure of polysaccharides represents a new and promising approach to certain problems in this field which appear to be incapable of solution by purely chemical means. The outstanding property of enzymes is their specificity of action and it is clear that the fullest possible use can only be made of this property if very pure enzyme preparations are available.

Such preparations have been used with great success in determining the fine structure of glucans, notably amylopectin and glycogen. No work of this kind on mannans has as yet been reported, though mannanases have been detected in malt extract (53,54), in the digestive juices of molluscs and crustaceans (55), and in Bacterium aroideae and Bacillus mesentericus (56).

Whistler and his co-workers (57) have obtained an enzyme preparation from germinating guar seed capable of hydrolysing guaran to the extent of about 65%. This preparation was later used in the preparation of a mannobiose (13) and a mannotriose (15), from guaran.

THE MANNANS OF IVORY NUT

Mannans constitute the reserve food supply of palm seeds and are stored in the endosperm in the form of greatly thickened cell-walls. In the seeds of Phytelephas macrocarpa, the tissue acquires a very hard, bonelike character which has led to its use in commerce under the name "vegetable ivory". This material was formerly much used for making buttons, but nowadays it has little commercial significance. The ivory nut is however a very convenient source of mannose.

The first important investigation of the structure

of the carbohydrates of ivory nut was made by Reiss (58) in 1889. He showed that the endosperm cell-walls were chiefly composed not of cellulose, as had previously been thought, but of a carbohydrate which he called seminin. This he obtained by extraction with cold 75% sulphuric acid. On hydrolysis the material yielded a syrup which Reiss termed seminose, but which was shown by Fischer and Hirschberger (59) to be identical with mannose. Later Johnson (60) showed that a mannan could be extracted from ivory nuts by extraction with dilute alkali and this was confirmed by a number of other workers (61,62,63).

It was not until 1923 however, that the methylation technique was applied to ivory nut mannan by Patterson (69). After a somewhat drastic pretreatment with hot 10% alkali he extracted a polysaccharide with 20% alkali. This was methylated with dimethyl sulphate and sodium hydroxide and the product on hydrolysis gave trimethyl mannose almost entirely. Since Patterson did not know the positions of the methoxyl groups in the sugar molecule, and was unable to determine whether the trimethyl mannose was a single substance or a mixture of isomers, he could make very few deductions concerning the structure of the polysaccharide.

Lüdtke (65) was the first to recognise that there are

apparently two mannans in ivory nut. He found that after delignification with chlorine dioxide and sodium sulphite, ivory nut shavings could be completely dissolved in cuprammonium hydroxide containing ammonium carbonate. The mannans were precipitated as a copper complex by the addition of sodium hydroxide, cellulose being left in solution. After decomposition of the complex with acetic acid the mannan mixture was separated into a component soluble in 4% alkali which Lüdtké called "Mannan A" and an insoluble residue which he termed "Mannan B". Mannan A was the same polysaccharide as previous workers had obtained by direct extraction with dilute alkali. Apart from their solubilities the two mannans differed in optical rotation and in the fact that Mannan B gave a colour with chlor-zinc-iodide while Mannan A did not.

Mannan A

Mannan A has been extensively studied by Klages (66). He delignified ivory nut shavings with chlorine di-oxide and extracted Mannan A with 5% sodium hydroxide. The mannan was methylated with dimethyl sulphate and sodium hydroxide at an elevated temperature (80°C), a rather extreme procedure by modern standards, and a water-soluble product was obtained which had a methoxyl content of 45.8%. On hydrolysis a trimethyl mannose was obtained which was shown to be identical

with the 2:3:6-tri-O-methyl-D-mannose of Haworth, Hirst and Streight (67). He concluded that the mannan was composed of 1:4-linked mannopyranose units. Klages also attempted to ascertain whether these linkages were of the α - or β - type, but obtained no conclusive results. He thought however, that both were present. The question was re-examined by Klages and Maurenbrecher in a later paper (68). They prepared a mannobiosazone from the partial hydrolysis products of the mannan, and from its properties concluded that a purely

-linked structure must be assigned to the polysaccharide. Chain length measurements by end-group assay suggested a degree of polymerisation (D.P.) of about 80.

Mannan A has also been studied by Ward (69). After hydrolysing the mannan and destroying the mannose by fermentation with yeast, he could not, with the techniques then available, detect galactose in the residue. The polysaccharide was methylated, hydrolysed, and the methylated sugars separated by fractional distillation of the glycosides. Two components, 2:3:4:6-tetra-O-methyl-D-mannose and 2:3:6-tri-O-methyl-D-mannose were identified, a second trimethyl mannose fraction was thought to contain 2:3:6- and possibly 2:3:4-tri-O-methyl-D-mannose. From these results and periodate oxidation studies Ward considered that the

mannan had a chain length of 15 and contained other linkages besides the 1:4.

Recently Aspinall, Hirst, Percival, and Williamson (70) have investigated the ivory nut mannans, making use of modern chromatographic techniques. Mannan A was isolated by extraction of delignified ivory nut shavings with 7% alkali and purified by precipitation as the copper complex. The product had a specific rotation of -46° in agreement with the value -45° reported by Klages (66). It gave mannose (97.6%), galactose (1.8%) and glucose (0.8%) on hydrolysis. Various attempts were made to determine the degree of polymerisation of the mannan; the results obtained by different methods differed considerably but appeared to indicate a value of 10-14. The polysaccharide was methylated and hydrolysed, and the sugars produced were separated by partition chromatography on a cellulose column. The results obtained are summarised in the table below:-

<u>Fraction No.</u>	<u>Contents of fraction</u>	<u>Molar ratios</u>
1.	2:3:4:6-tetra- <u>O</u> -methyl-D-mannose	4.3
2.	2:3:4:6-tetra- <u>O</u> -methyl-D-galactose	1
3.	2:3:6-tri- <u>O</u> -methyl-D-mannose	49
4.	2:3:4-tri- <u>O</u> -methyl-D-mannose	4.0
5.	2:3-di- <u>O</u> -methyl-D-mannose	0.7

Fraction 4, described here as 2:3:4 trimethyl D-mannose was obtained as a syrup which later crystallised and became non-reducing to Fehling's solution. Examination of this crystalline substance indicated that it was a 1:1-linked, partially methylated disaccharide which had formed (presumably) by the self condensation of two molecules of 2:3:4-tri-O-methyl-D-mannose.

From these results the following alternative structures for Mannan A were postulated:-

1. The polysaccharide is a mixture of two molecular species:-

- (a) a linear molecule of 10 anhydro-D-mannopyranose units linked through carbon atoms 1 and 4, and one residue with a 1:6 linkage;
- (b) a linear molecule of 10 anhydro-D-mannopyranose units linked through carbon atoms 1 and 4 and terminated at the non-reducing end by a D-galactopyranose residue.

The ratio of (a) to (b) would have to be 4:1 approximately.

2. The polysaccharide is a mixture of two molecular species:-

- (a) a linear molecule of about 13 mannose residue

linked through carbon atoms 1 and 4;

- (b) a linear molecule of 4 mannose residues linked through carbon atoms 1 and 6, terminated at the non-reducing end by a galactose residue.

Mannan B

Ivory nut Mannan B has been investigated by Klages who isolated it by the method described by Ludtke (65). The mannan was methylated with difficulty and gave on hydrolysis 2:3:4:6-tetra-O-methyl-D-mannose and 2:3:6-tri-O-methyl-D-mannose. Klages considered that Mannan B differed somewhat in constitution from Mannan A because of differences in the optical rotation of the polysaccharides and their acetyl and methyl derivatives. He thought that the difference might lie in the ratio of α - to β -linkages.

Aspinall, Hirst, Percival, and Williamson (7) have also studied Mannan B. They used essentially the same method as Ludtke to extract the crude polysaccharide from the residues left after the extraction of Mannan A. The product gave a considerable quantity of glucose as well as mannose on hydrolysis, but was purified by extraction with anhydrous formic acid in which the mannan is readily soluble. The purified mannan gave the same sugars on hydrolysis as Mannan A

in almost the same proportions, namely, mannose (98.5%), galactose (1.1%) and glucose (0.8%). The results obtained by methylation, hydrolysis, and separation of the products on cellulose are shown below:-

<u>Fraction No.</u>	<u>Contents of fraction</u>	<u>Molar ratios</u>
1.	2:3:4:6-tetra- <u>O</u> -methyl-D-mannose	1
	2:3:4:6-tetra- <u>O</u> -methyl-D-galactose	1
2.	2:3:6-tri- <u>O</u> -methyl-D-mannose	63
3.	2:3:4-tri- <u>O</u> -methyl-D-mannose	11
4.	2:3-di- <u>O</u> -methyl-D-mannose	1

Fraction 3 had the same properties as the corresponding fraction obtained from Mannan A.

From these results it was suggested that two structures were possible for the mannan:-

1. an equimolecular mixture of two types of linear molecules, one having a mannose and the other a galactose end-group;
2. branched structures containing mannose and galactose non-reducing end-groups in the same molecule.

As with Mannan A the estimation of the D.P. of the mannan by different methods gave varying results, but a value of about 38 for a straight-chain structure and at least 76 for a branched-chain structure.

It is clear that considerable uncertainty still exists concerning the fine structure of the ivory nut mannans. The object of the present investigation was to attempt to obtain more information about the structure of these polysaccharides by carrying out partial hydrolyses and identifying the oligosaccharides produced.

In addition a preliminary investigation has been made of a mannan or glucomannan isolated by Dr. R.A. Laidlaw from the wood of the Sitka Spruce.

THE MANNANS OF IVORY NUT

DISCUSSION

MANNA A

Ivory nut shavings were extracted with benzene and methanol and delignified by treatment with sodium chlorite and acetic acid at 70-80°C. The delignified shavings were shaken with 7% potassium hydroxide solution and Mannan A precipitated with ethanol from the acidified extract. The product had $[\alpha]_D^{18} -48^\circ$ (C, 1.13 in N sodium hydroxide), compared with the values $[\alpha]_D^{18} -46^\circ$ reported by Aspinall, Hirst, Percival, and Williamson (70) and $[\alpha]_D^{20} -45^\circ$ reported by Klages (66). On hydrolysis the mannan yielded the following sugars, which were identified on a paper chromatogram:- mannose, xylose, and traces of glucose and galactose. Aspinall, Hirst, Percival, and Williamson purified the crude mannan by precipitating it as the copper complex with Fehling's solution. This procedure removed traces of pentosan impurity. It was considered that purification was not essential in the present work, since under the conditions employed for the partial hydrolysis of the mannan the pentosan would be completely converted to monosaccharide. This assumption was later found to be correct.

Because of its low solubility, Mannan A is hydrolysed only very slowly in hot dilute mineral acid. The polysaccharide was therefore subjected to a graded acetolysis, a process of simultaneous acetylation and hydrolysis, by treatment for

72 hours, at room temperature, with a mixture of acetic anhydride, glacial acetic acid, and concentrated sulphuric acid. The mixture was poured into ice-water, and the precipitated acetates washed and dried. Saponification of the acetates was effected by treating the solution in absolute methanol with a catalytic amount of barium methoxide. This method has the advantage that free alkali can never be present in the solution; any barium hydroxide which may be formed by traces of moisture is at once precipitated. Moreover barium is readily removed by the addition of an equivalent volume of dilute sulphuric acid.

The oligosaccharide mixture was fractionated on a Charcoal-Celite column in the manner described by Whistler and Durso (16). Monosaccharides were eluted with water. Elution with 5% aqueous ethanol yielded a fraction which appeared to consist of a single, chromatographically pure, sugar, having an R_M value (C.O.5) corresponding to a disaccharide. The sugar gave mannose only, on hydrolysis, and was considered to be β -1:4 manno-biose, a disaccharide which has been isolated by Whistler and Stein (13). Attempts to crystallise the sugar by the method used by these workers were at first unsuccessful. A portion of the syrup was

therefore methylated, first with dimethyl sulphate and sodium hydroxide solution and then with silver oxide and methyl iodide, and the product hydrolysed.

Examination of the hydrolysate on a paper chromatogram indicated the presence of a tetra- and a tri-O-methyl mannose. The two components were separated chromatographically on thick paper and shown, by preparation of their aniline derivatives, to be 2:3:4:6-tetra-O-methyl-D-mannose, and 2:3:6-tri-O-methyl-D-mannose.

Periodate oxidation studies were also carried out on the disaccharide. Oxidation with sodium metaperiodate, and measurement of the periodate uptake, showed that 4 moles of periodate per mole of disaccharide were consumed very rapidly; this uptake corresponded, presumably, to scission between C₁, C₂, and C₃ at the reducing end of the molecule, and between C₂, C₃ and C₄ and the non-reducing end. Thereafter oxidation proceeded more slowly; there was no definite end-point at the theoretical uptake of 5 moles. For the determination of the formic acid released the disaccharide was oxidised with potassium periodate since this method minimises over-oxidation (74,75). It was found however that there was a continuous evolution of formic acid over and above the theoretical limit of 3 moles per mole.

Later the disaccharide was crystallised, originally by the method of Whistler and Stein and afterwards from ethanol/water. The mannobiose melted at a temperature several degrees higher than that reported by these workers and it mutarotated downwards and not upwards. Comparison of the X-ray powder photographs has shown that the two substances are identical; it is possible that the sugar forms mixed crystals containing varying proportions of the α - and β - forms.

A fraction which was also eluted with 5% ethanol appeared to consist of a mixture of mannobiose with two other disaccharides. Attempts to separate this mixture on charcoal and cellulose columns and chromatographically, on thick paper, were unsuccessful.

The third fraction, which was eluted with 7% aqueous ethanol consisted of a mixture of mannobiose and a sugar which appeared to be trisaccharide. The mixture was re-fractionated on charcoal and the trisaccharide isolated in a chromatographically pure condition. The pure sugar gave mannose on complete hydrolysis, and mannobiose and mannose on partial hydrolysis. The trisaccharide crystallised readily, as the trihydrate, from hot aqueous ethanol. and after several recrystallisations it melted at 134.5-135.5°C.

and had $[\alpha]_D^{19} -20.2^\circ$ (equilibrium value). Whistler and Smith (15) give the melting-point of β -1:4 mannotriose as $137-137.5^\circ\text{C}$ and the specific rotation as -23.3° (equilibrium value), these values are in fairly close agreement with those quoted. Comparison of the X-ray powder photographs of the two sugars has shown that they are identical. As in the case of the disaccharide the direction and magnitude of the mutarotation differ from those reported by Whistler and Smith. These authors prepared a crystalline mannotritol acetate from the trisaccharide by catalytic reduction with hydrogen followed by acetylation. Under the same conditions the mannotriose was reduced only very slowly. Reduction with potassium borohydride at first gave unsatisfactory results. Using conditions similar to those employed by Abdel-Akher, Hamilton and Smith, and a reaction period of several hours, it was found that considerable degradation appeared to occur giving a number of products including mannitol. Hough, Jones and Richards (77) have reported similar results in the prolonged reduction of lactose with borohydride. More recently, Whelan and Morgan (78) have found no evidence for the degradation of lactose during borohydride reduction. This prompted a further attempt to reduce the mannotriose by

this method. Using a large excess of potassium borohydride, reduction was effected in 40-60 minutes. The glycitol was acetylated in the usual way and the mannotriitol acetate was found to have a melting-point and optical rotation similar to those reported.

It has been found that the trisaccharide melts at two different temperatures depending on the rate of heating. The anhydrous sugar melts above 160°C , but on fairly rapid heating, or by heating in a sealed tube, the hydrate melts at 130°C . Another similar phenomenon has been described by Whistler and Corbett (79) in the case of 2-O-D-xylosyl-L-arabinose.

Elution of the column with 10% aqueous ethanol gave a mixture of mannotriose with two other trisaccharides, and mannobiose. The mixture was successfully fractionated on a cellulose column, using ethyl acetate-pyridine-water as solvent, and chromatographically pure samples of the anomalous trisaccharides were obtained. The yields however were small.

A further quantity of Mannan A was subjected to a graded acetolysis in the hope that sufficient quantities of the anomalous oligosaccharides could be isolated to enable them to be characterised. The method used for the acetolysis was the same as before except that after the mixture had been

poured into water and the solid acetates separated, the mother liquor was extracted with chloroform. This resulted in a much higher recovery. The acetates were deacetylated with barium methoxide and the mixture of free sugars fractionated on a Charcoal-Celite column. The procedure employed differed in several respects from that already described. A much lower loading was employed (20 g. charcoal/g.mixture, as compared with 9g./g.), and the ratio of charcoal to celite (Grade 545) was increased to 2:1 to keep the dimensions of the column within reasonable limits. The concentration of ethanol in the eluant was increased in steps of 1% or 0.5%, this method has some of the advantages of gradient elution while enabling the rate of elution to be controlled. As before the first five fractions consisted of monosaccharides, mannobiose, mixed disaccharides, mannotriose and mixed trisaccharides. Elution was continued and a further fraction was obtained containing a sugar which later experiments indicated as being β -1;4 mannotetraose. The next fraction contained as its principal component a sugar which ran faster than mannotetraose on a paper-chromatogram although it was eluted after it from charcoal. Two more fractions were obtained which corresponded exactly to the

previous two in that the first contained mannopentaose (presumably) and the second a pentasaccharide with a higher chromatographic mobility. It seems probable that still higher oligosaccharides could be eluted provided a suitable solvent could be developed for following the elution chromatographically.

The separation of the β -1:4-linked oligosaccharides was very good and all were sufficiently pure to be crystallised directly. Further purification was effected by recrystallisation.

The fractions containing mixtures of disaccharides and trisaccharides were refractionated on cellulose columns using a solvent developed for this purpose, ethyl acetate-isopropanol-water (16:10:5 v/v). This solvent gave good separations, has a fairly high mobility, and has the advantage of not containing pyridine.

Disaccharide B, which has the highest chromatographic mobility of the three disaccharides present, gave mannose on hydrolysis, and, since it had a high positive optical rotation (+ 49°) was clearly α -linked. When oxidised with sodium periodate in bicarbonate buffer it gave formaldehyde, showing that the linkage was not 1:5 or 1:6. The disaccharide formed an osazone, which was not glucosazone,

when heated with phenyl hydrazine acetate in the usual way, showing that it could not be linked in the 2-position. Perlin (80) has shown that disaccharides oxidised with lead tetraacetate react initially in the ring form. In 1;3-linked hexose disaccharides scission occurs very rapidly between C₁ and C₂ at the reducing end of the molecule. The rate of oxidation then decreases very markedly owing to the formation of formyl esters. If the oxidation is stopped at this stage and the product hydrolysed, a pentose is formed. With 1;4 disaccharides scission also occurs between C₂ and C₃ with the result that a tetrose is produced on hydrolysis. Disaccharide B (isomannobiose) was oxidised in the manner described by Perlin and after hydrolysis the products were examined on a paper chromatogram. Only tetrose was detected. The disaccharide is slightly soluble in dilute methanolic hydrogen chloride and its rotation in this solvent was unchanged after 6 hours. These results all suggest that the disaccharide is 4-O- α -D-mannopyranosyl-D-mannopyranose.

Disaccharide C, which moved more slowly than mannobiose on a paper chromatogram, gave mannose and glucose on hydrolysis. The disaccharide had a low positive optical rotation, gave formaldehyde on periodate oxidation and

on oxidation with lead tetraacetate, followed by hydrolysis, a tetrose. It also formed an osazone. The disaccharide was oxidised with bromine water and hydrolysed. The hydrolysate was examined on a paper chromatogram and it was found that mannose was the only reducing sugar present.

These results indicate that the disaccharide is a β -1:4-linked mannosyl glucose. Its osazone however is not identical with that prepared from mannobiose. Moreover the disaccharide is more readily hydrolysed than mannobiose which would not be expected if the mode of linkage were the same in both cases. A decision as to the linkage in this disaccharide must therefore await further evidence.

Trisaccharide X bore the same chromatographic relation to mannotriose as isomannobiose did to mannobiose. On complete hydrolysis it gave only mannose, and on partial hydrolysis a mixture of mannose, mannobiose and isomannobiose. The trisaccharide was reduced with potassium borohydride and the sugar alcohol subjected to partial hydrolysis. The only reducing sugars detected on a paper chromatogram were mannose and isomannobiose. The anomalous link is therefore at the reducing end of the molecule. The trisaccharide was crystallised from ethanol/water. After recrystallisation the crystals melted at 224-225°C and had $[\alpha]_D^{25} + 40^\circ$ (C, 2.0 in water, equilibrium value) thus confirming the presence of an α -link

in the sugar. Isomannotriose thus appears to be α -D-mannopyranosyl-(1 \rightarrow 4)- β -D-mannopyranosyl-(1 \rightarrow 4)-D-mannopyranose.

Trisaccharide Y, which had a lower chromatographic mobility than mannotriose, gave mannose and glucose in the ratio 2:1 on hydrolysis. Partial hydrolysis gave a mixture of the monosaccharides with mannobiose and mannosyl glucose. The trisaccharide was reduced with borohydride and partially hydrolysed. A paper chromatogram of the hydrolysate showed that mannose and mannobiose were the only reducing sugars present; hence the glucose residue must form the reducing end of the molecule. The trisaccharide had a low negative optical rotation as would be expected if both links are of the β -type.

The "mannotetraose" and "mannopentaose" fractions were crystallised from ethanol/water and some evidence has been obtained from their postulated structures. On hydrolysis they give only mannose; on partial hydrolysis mannotetraose yields mannose, mannobiose, and mannotriose, while mannopentaose gives the same sugars and mannotetraose also. The molecular rotations (equilibrium values) of the series mannobiose \longrightarrow mannopentaose fall on a straight line when plotted against chain-length (105). Again

a plot of $\log \left(\frac{R_M}{1-R_M} \right)$ (89) against chain-length gives a reasonably straight line, bearing in mind the fact that the low R_M value (c.0.01) of mannopentaose, makes accurate measurement difficult.

The anomalous tetraose and pentaose fractions have been only partially investigated. A sample of chromatographically-pure pentasaccharide has been isolated and found to give only mannose on hydrolysis. On partial hydrolysis mannose, mannobiose, isomannobiose (a trace), mannotriose, mannotetraose, and a spot having the same R_M as the anomalous tetrasaccharide are produced. This suggests that the sugar contains three adjacent β -1:4 links and an α -1:4 link at the non-reducing end, and that these anomalous oligosaccharides are homologues of isomannobiose and isomannotriose.

In assessing the significance of the results obtained several factors must be borne in mind. The first is that random hydrolysis of a polysaccharide containing different types of linkage, of varying acid lability, inevitably gives a distorted picture of the distribution of the various linkages. Secondly the possibility of reversion and epimerisation must be considered.

There can be no doubt however that the present work confirms the earlier methylation studies in that there is ample evidence that Mannan A is composed of chains of mannose

residues linked predominantly by β -glycosidic links in the 4 position. No galactose-containing oligosaccharides have been isolated suggesting that the galactosidic link must be weaker than the others present in the molecule. The isolation of what appears to be a homologous series of mannose oligosaccharides containing an α -1:4 link at the non-reducing end suggests that this link is a structural feature of the mannan and occurs singly at intervals in the chains. On the other hand the significance of the glucose-containing sugars is more difficult to determine. The possibility that they have arisen by epimerisation of the corresponding mannose oligosaccharides cannot be entirely ruled out although it seems unlikely.

MANNAN B

Mannan B was extracted from the residues of the Mannan A extraction by the method described by Aspinall, Hirst, Percival and Williamson (70). The residues were dissolved in cuprammonium hydroxide solution and the crude mannan precipitated as its copper complex by the addition of sodium hydroxide solution. The complex was decomposed with acetic acid and the product freed from Mannan A by extraction with 7% potassium hydroxide solution. The residue was extracted with formic acid and the extract poured into ethanol. The precipitate was considered to be pure Mannan B: it had $[\alpha]_D^{25} -26^{\circ}$ (c 1.1 in anhydrous formic acid) which is the same value as that reported by the above workers.

The polysaccharide was subjected to acetolysis under the same conditions employed for Mannan A except that a longer reaction period (96 hours) was used. The products were deacetylated, and separated on a Charcoal-Celite column. Mannobiose, mannotriose, and mannotetraose, were isolated in a pure condition, and crystallised. Isomannobiose, isomannotriose, mannosyl glucose, and the corresponding trisaccharide were all found to be present. It therefore seems likely that Mannan A and Mannan B are essentially similar in structure, the differences between them must be due mainly to a difference in the molecular size.

EXPERIMENTAL

GENERAL METHODS

Chromatography

All separations on paper were made using descending chromatography on Whatman No. 1 paper, unless otherwise stated. When continuous elution over a long period was required the lower edge of the paper was serrated to allow the solvent to drip off evenly.

The solvents employed were as follows:-

n-butanol-benzene-pyridine-water (5:3:3:1 v/v top layer)

ethyl acetate-pyridine-water (10:4:3)

n-butanol-ethanol-water (4:1:5 top layer)

benzene-ethanol-water (170:50:15 top layer)

methyl-ethyl-ketone-water-saturated.

For separating free sugars n-butanol-pyridine-benzene-water was used in the early stages of this work. Later it was found that equally good separations could be obtained in a very much shorter time by employing ethyl acetate-pyridine-water (18 hours for monosaccharides). This solvent was allowed to stand at room temperature for at least 24 hours before use to allow for equilibration.

The spray reagent employed for reducing sugars was saturated aqueous aniline oxalate and the papers were developed at 150° C for 3 - 5 minutes.

Distillation All distillations of aqueous solutions were carried out at 40°C under reduced pressure.

Hydrolysis The hydrolysates from formic acid hydrolyses were treated as follows - the formic acid was distilled off under reduced pressure, water was added and the distillation repeated several times in order to remove the last traces of formic acid as the azeotrope. The residues were taken up in N sulphuric acid and heated, usually in sealed tube at 100°C for 3 hours to hydrolyse formyl esters. The solutions were then cooled, neutralised with barium carbonate and concentrated to a small volume under reduced pressure.

Methoxyl content was determined by a volumetric micro-modification of the Zeisel method.

MANNNAN A

Extraction of Mannan A

Ivory nut shavings were extracted, first with benzene and then with methanol, for 16 hours each, in a Soxhlet extractor, to remove waxes, colouring material etc. The extractives - free material, was then delignified by the method of Wise, Murphy and d'Addieco (81). The shavings (100 g) were stirred with 3 litres of hot water and sodium chlorite (30 g) and glacial acetic acid (5 ml) were added. The mixture was heated at 70-80°C., with occasional stirring, for one hour, when a further 30g. of sodium chlorite and 5 ml. of acetic acid were added. The mixture was then heated for another hour. Two further additions of the reagents were made in the same way so that in all the mixture was heated for 4 hours. After cooling, the shavings were filtered off through muslin, washed with ice-water and dried by suction.

The cleaned shavings were shaken for 18 hours with 7% potassium hydroxide solution (2-3 litres). The residual material was removed by centrifugation, and the yellowish solution acidified with glacial acetic acid. The addition of an equal volume of methylated spirits precipitated the mannan which was washed with water and methylated spirits and dried with ethanol and ether.

A small sample (c.20 mg) of the mannan was hydrolysed

by heating for 7 hours in a sealed tube with anhydrous formic acid (1ml.). Examination of the hydrolysate by paper chromatography showed that the main product was mannose, together with very small amounts of galactose and glucose, and a pentose, probably xylose. The mannan, which was obtained as a white powder, had $[\alpha]_D^{18} -48^\circ$ (c, 1.1 in N sodium hydroxide). The yields were much lower than those reported by Williamson (82) and averaged about 12-15% of the weight of starting material.

Graded Acetolysis of Mannan A (1)

The mannan was subjected to acetolysis by the method described by Dickey and Wolfrom (83). Previously, small-scale experiments had shown that a mixture yield of di- and tri-saccharides was obtained after about 72 hours treatment with the acetolysis mixture. Accordingly, Mannan A (75 g.) was added slowly, with stirring, to a mixture of acetic anhydride (450 ml.), glacial acetic acid (450 ml.), and concentrated sulphuric acid (45 ml.), cooled to 0°C in a freezing mixture. The temperature rose only a few degrees during the addition of the mannan. The mixture was allowed to warm up slowly to room temperature and stand for 72 hours. The mannan dissolved completely after about 36 hours. The

solution, which was yellowish-brown in colour with a green fluorescence, was filtered through sintered glass and poured slowly, with stirring, into 3 litres of ice-water. Sodium bicarbonate was added till the pH of the solution was 3-4, and the mixture was allowed to stand overnight. The rather sticky precipitate was filtered off and washed thoroughly with water, when it hardened and became easier to handle, and dried in vacuo over sodium hydroxide. Yield 72 g.

Deacetylation of the acetolysate

The acetates were deacetylated by the catalytic barium methoxide method of Isbell (84). The mixture (72 g.) was dissolved as far as possible in absolute methanol (400 ml.), cooled to 0°C in a freezing mixture. Barium methoxide solution (40 ml, 0.5N), prepared by adding barium oxide to absolute methanol and filtering off the barium hydroxide), was added, and after a few seconds a considerable curdy precipitate formed. The mixture was kept in the cold and shaken at intervals for an hour, and then allowed to stand in the refrigerator overnight. The mixture was tested with phenolphthalein for excess barium methoxide and then slightly less than the equivalent volume of dilute (0.5N) sulphuric acid was added with vigorous shaking, followed by water (300 ml). The mixture was well shaken to dissolve the free sugars and

centrifuged to remove barium sulphate and a small amount of insoluble carbohydrate material. The solution was evaporated to a syrup which was dried in vacuo over sodium hydroxide. Yield 38.0g.

Separation of oligosaccharides on a Charcoal-Celite Column

A column 75 x 520 mm. was prepared from 350g. each of M & B decolourising charcoal and Johns-Manville Celite (grade Hyflo-Supercel). The Celite was washed with dilute Hydrochloric Acid (1:1 v/v) and with water by decantation until free from acid, and the charcoal was washed with water several times by decantation. The two components were mixed into a slurry, poured into the column and allowed to settle and washed again with several litres of water. The sugars (38g.) were dissolved in water (150 ml.) and absorbed onto the column. Elution was then begun with water and continued with aqueous ethanol in the concentrations 5%, 7%, 10%, 15%, 20%, and 50%. The effluent was collected in litre fractions which were evaporated to dryness and examined by paper chromatography, using ethyl acetate-pyridine-water as the solvent. The original fractions were combined into six main fractions;

Fraction 1 (3.3g) This fraction was eluted with water and contained mannose, galactose, glucose and pentose.

Fraction 2 (6.1g) Eluted with 5% aqueous ethanol. This fraction appeared to consist of a single, chromatographically pure, sugar which travelled on a paper chromatogram at a rate corresponding to a disaccharide ($R_M, 0.52$) On hydrolysis (3 hours in N sulphuric acid at 100°C) it gave only mannose. Since the 1:4 glycosidic link is known to be predominant in the molecule of Mannan A it was anticipated that this sugar would be identical with the β -1:4 mannobiose isolated and crystallised by Whistler and Stein (13). Attempts to crystallise the disaccharide by the method employed by these workers were for a long time unsuccessful. The mannobiose was therefore methylated and subjected to periodate oxidation.

Methylation of Mannobiose

A sample (1 g.) of the mannobiose was methylated by the method of Haworth and Leitch (85). The sugar was dissolved in a minimum volume of water, dimethyl sulphate (8 ml.) was added and then 30% sodium hydroxide solution (5 ml.) was added very slowly with continuous stirring. The mixture was stirred overnight and then a further 15 ml. of sodium hydroxide solution was added slowly. After stirring for 1 hour the temperature was raised to 55°C and further portions of dimethyl sulphate (8 ml.) and sodium hydroxide (20 ml)

were added slowly with stirring over a period of 4 hours. The whole operation was carried out in a stream of nitrogen. The solution was cooled, neutralised with glacial acetic acid and extracted with chloroform. The extract was dried over calcium chloride and the solvent evaporated.

The resulting syrup was dissolved in methyl iodide (8 ml.), silver oxide (0.5 g) was added and the mixture refluxed for half an hour. Seven further additions of silver oxide were made in the same way. Methyl iodide was distilled off and the residue extracted with chloroform. The chloroform extract was concentrated to a syrup. Yield 310 mg.

Hydrolysis of the methylated mannobiose

A portion (100 mg.) of the methylated disaccharide was hydrolysed by heating for six hours in N sulphuric acid in a sealed tube at 100°C. The solution was cooled and neutralised with barium carbonate. A paper chromatogram of the hydrolysate (solvent, n-butanol-ethanol-water) showed the presence of two components in approximately equal proportions. Component A gave a pink spot with aniline oxalate and moved at the same rate as 2:3:4:6 tri-O-methyl-D-mannose; Component B gave a brown spot and had the same chromatographic mobility as 2:3:6 tri-O-methyl-D-mannose. Separation of the two components was effected on Whatman 3 MM paper. The paper was irrigated

with n-butanol-ethanol-water and the positions of the sugars found by cutting off side-strips which were sprayed and heated in the usual way. The portions containing the sugars were cut off, extracted with acetone in a Soxhlet extractor, and the acetone solutions evaporated under reduced pressure.

Yields: Component A: 35 mg.

Component B: 25 mg.

Characterisation of the hydrolysis products

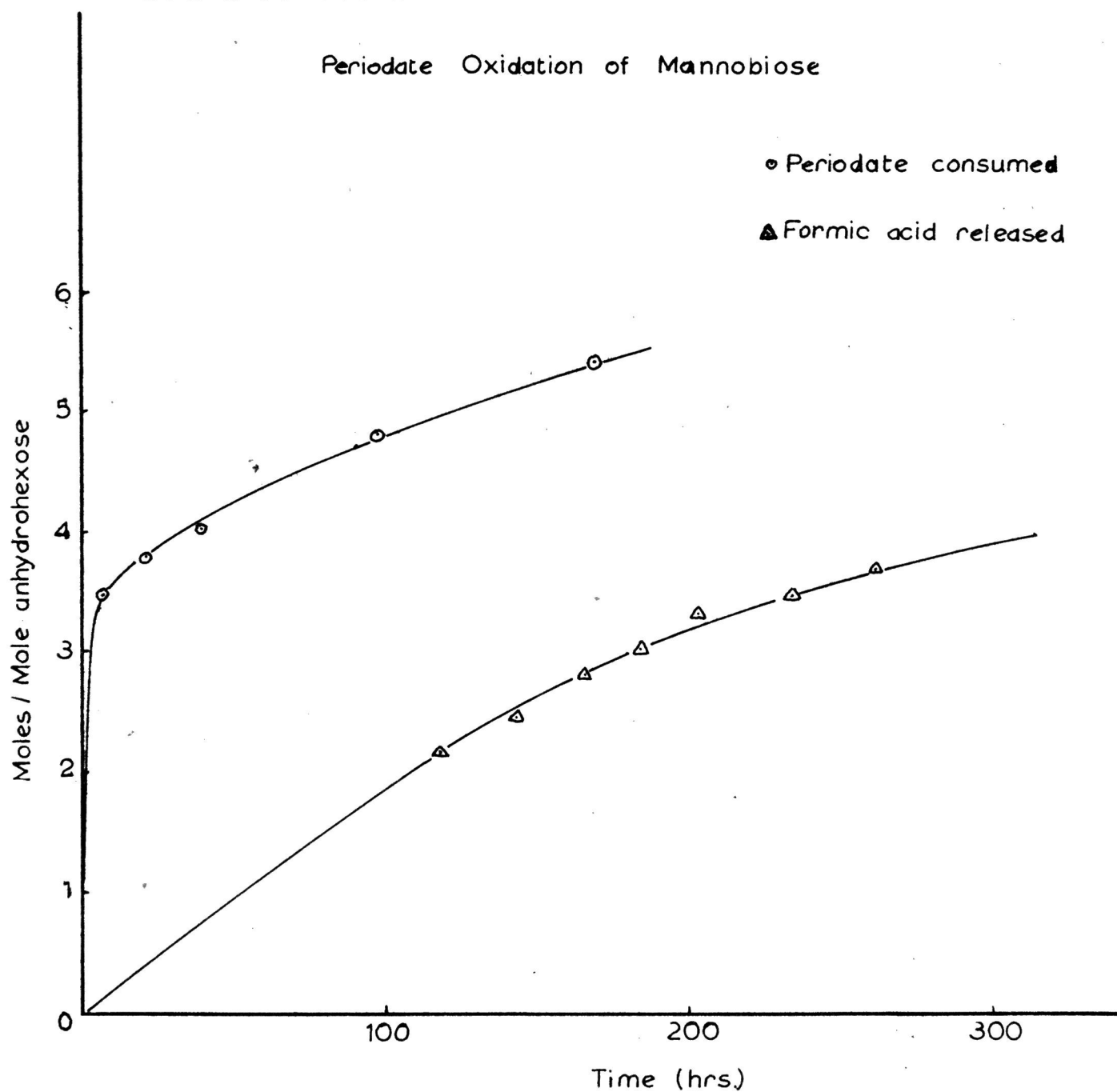
The methylated sugars, which were obtained as syrups, were converted to the aniline derivations by heating with the calculated weight of freshly-distilled aniline for 2 hours in absolute alcohol. The solvent was removed under reduced pressure leaving a crystalline residue which was washed with a small quantity of a mixture of ether and light petroleum (B.P. 100-120°C) and re-crystallised from ether.

The anilide of Component A melted at 141-143°C undepressed on admixture with an authentic sample of 2:3:4:6-tetra-O-methyl-N-phenyl-D-mannosylamine. This agrees with the value 142-143°C reported for this compound by Irvine and MacNicholl (86).

The aniline derivative of Component B melted at 120.5 - 123°C, this is rather low compared with the value 127-128°C given by Haworth et al. (87) for 2:3:6 tri-O-methyl-N-phenyl-

GRAPH No.1.

Periodate Oxidation of Mannobiose



D-mannosylamine, but was undepressed on admixture with an authentic sample of this compound.

Periodate oxidation of Mannobiose

1. Determination of periodate uptake A sample (48.38 mg.) of the mannobiose was dissolved in water (50 ml.) and 0.3 M sodium metaperiodate solution (10 ml.) was added. The mixture was allowed to stand in a well-stoppered bottle in the dark and at intervals 10 ml. samples were withdrawn and treated as follows. Sodium bicarbonate (c. 1g.) was added followed by a known excess of 0.1N sodium arsenite solution and potassium iodide (1g.). After standing for 15 minutes the excess arsenite was estimated by titrating with 0.0997N iodine solution. A reagent blank was carried out at the same time, the difference between the two titrations is equivalent to the periodate consumed.

Results

<u>Time</u> <u>(hours)</u>	<u>Titre</u> <u>(ml.)</u>	<u>Periodate uptake</u> <u>Moles/C₁₂H₂₂O₁₁</u>
7	1.64	3.47
22	1.79	3.79
50	1.91	4.04
98	2.28	4.81
170	2.55	5.39

2. Determination of formic acid released The formic acid released was determined by the method of Halsall, Hirst, and Jones (88) as modified by Chanda, Hirst, Jones and Percival (89). A sample of the disaccharide (87.93 mg.) was dissolved in 100 ml. water in a Quickfit flask and potassium chloride (5 g.) and 0.3M sodium metaperiodate solution (20 ml.) added. The solution was shaken in the dark; at intervals the flask was removed from the shaker and a 10 ml. aliquot withdrawn. The solution was treated with ethylene glycol (1 ml.) to destroy excess periodate, and titrated with 0.01060 N sodium hydroxide solution (carbonate-free) using methyl red as indicator. A blank experiment was also run and the values of the blanks subtracted from the original titres.

Results

<u>Time (hours)</u>	<u>Titres (ml.)</u>	<u>Moles HCOOH/ Mole C₁₂H₂₂O₁₁</u>
119	4.38	2.15
143	4.94	2.43
166	5.67	2.79
185	6.08	2.99
214	6.70	3.30
235	6.85	3.37
263	7.42	3.65
288	7.79	3.83

Crystallisation of Mannobiose

The disaccharide was first crystallised by the method of Whistler and Stein (13). A portion of the syrup was dissolved in a small quantity of water, a considerable volume of methanol was added followed by n-butanol until the solution just became cloudy. The mixture was heated on the steam-bath until flocculation occurred, and allowed to cool. On standing for a long period at room temperature the sugar crystallised in spherulitic aggregates of needles. After re-crystallisation the sugar melted at 202-203°C. The remainder of the syrup could not be crystallised by this method but was crystallised as follows. The mannobiose was dissolved in a small volume of water and the solution heated on a boiling water-bath. Ethanol was added until heavy precipitation occurred: the mixture was kept at 35-40°C and crystallisation began after one or two hours. The amorphous material appeared to go into solution as crystallisation proceeded. The sugar crystallised in the same form using either method, as was shown by comparison of melting-points and X-ray powder photographs. After several re-crystallisations the mannobiose melted at 202-203°C and had $[\alpha]_D^{25} -5.2^\circ$ (extrapolated to zero time), changing after 2 hours to -8.5° (equilibrium value), (C.5.4).

Whistler and Stein (13) report that β -1:4 manno-
biose melts at 193.5° - 194° C and has $[\alpha]_D^{25}$ -7.7° (initial value)
changing to -2.2° (equilibrium value). A comparison of the
X-ray powder photographs of the two sugars by Professor
Whistler has shown that they are in fact identical. The
discrepancy in the physical properties has not been
explained.

The disaccharide is therefore 4-O- β -D-mannopyranosyl-
-D-mannopyranose.

Fraction 3 (1.8g.) (5% ethanol) This fraction contained
mannobiose together with two other sugars, one of which
travelled slightly faster on a paper chromatogram than
mannobiose (R_M 0.62)(solvent, ethyl acetate-pyridine-water)
and the other which travelled slightly slower (R_M 0.38)
than the latter sugar. The faster-moving sugar was
designated Disaccharide B and the other Disaccharide C.
Traces of monosaccharides were also present, together with
a considerable quantity of a trisaccharide.

A number of attempts were made to isolate disaccharides
B and C from this mixture. Small-scale experiments showed
that separation by paper chromatography on sheets of Whatman
3 MM paper would be unsatisfactory because of the high
proportion of manno-
biose present. Fractionation on a

charcoal-celite column, using a gradient elution technique (90) succeeded in reducing the amount of impurities and the proportion of mannobiose present. Attempted separations on cellulose columns were also unsuccessful, principally because of the difficulty of absorbing the sugars on to the column, their low solubility in the solvent mixture (ethyl acetate-pyridine-water) made the usual method impracticable. Application of the sugars in pyridine/water resulted in a very imperfect separation.

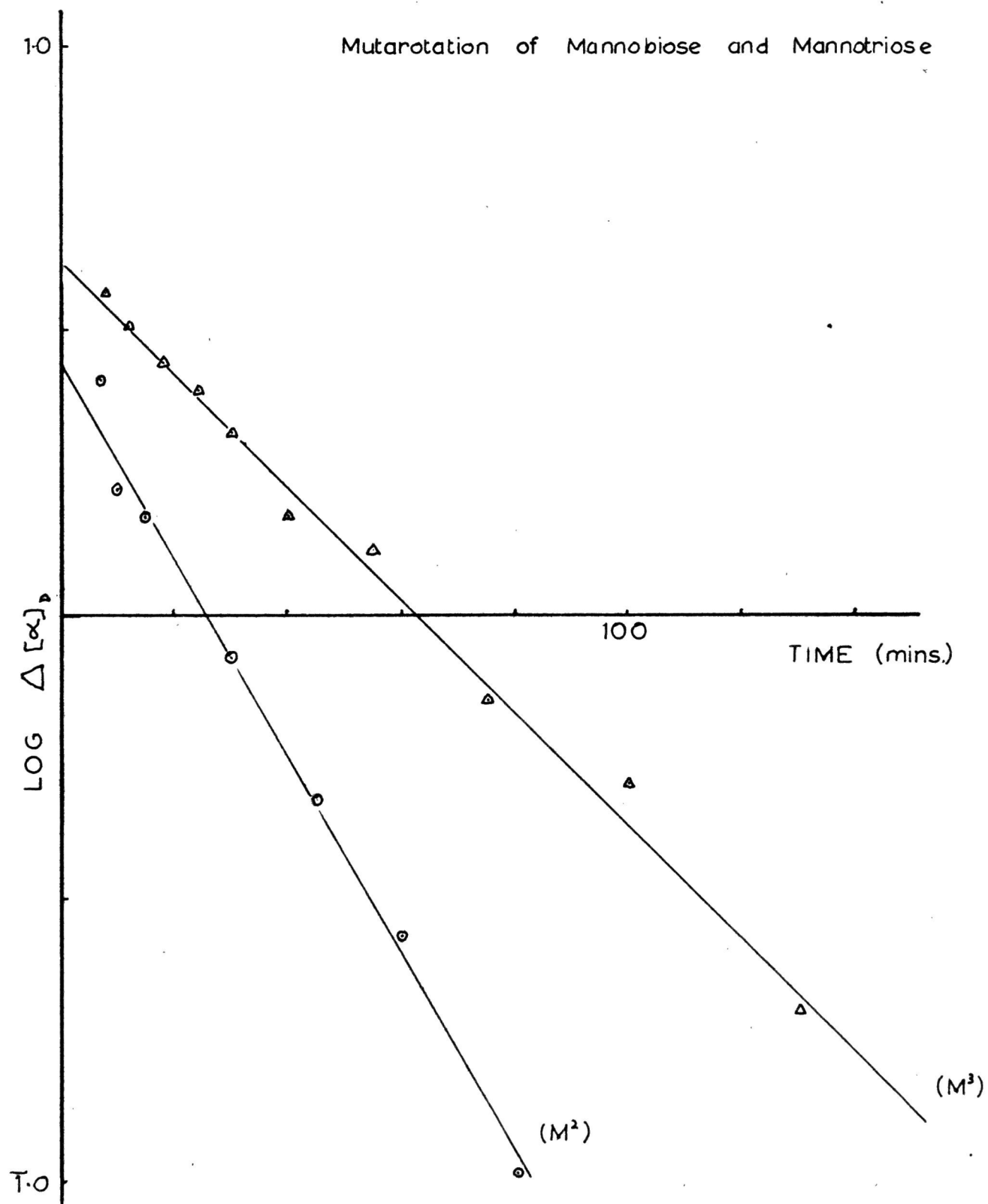
A separation on thick paper gave two fractions in poor yield (0.50mg.) which were still contaminated with mannobiose.

Fraction 4 (5.5 g.) This fraction, eluted with 7% aqueous ethanol, contained mannobiose and a sugar which was tentatively identified from its chromatographic mobility on charcoal and paper (R_M 0.22) as a trisaccharide. The components were separated by refractionating twice on charcoal-celite columns (28 x 260 mm.) using aqueous ethanol in the concentrations 5, 10 and 15 per cent. Fractions of 250 ml. were collected and those containing the trisaccharide were combined and evaporated to dryness. In this way 1.5 g. of the chromatographically pure sugar were obtained. Total hydrolysis of the sugar (3 hours in N sulphuric acid at 100°C)



GRAPH No. 2.

Mutarotation of Mannobiose and Mannotriose



gave only mannose. Partial hydrolysis (0.5N sulphuric acid at 100°C for 1 hour) gave mannobiose and mannose. The trisaccharide thus appeared to be the β -1:4 mannotriose.

Crystallisation of mannotriose The syrup was dissolved in a small volume of water and heated on a boiling water-bath. Ethanol was added slowly with stirring until the solution showed slight cloudiness. The liquid was then allowed to cool slowly when the sugar crystallised very readily in the form of plates. The melting-point of the first crop of crystals appeared to be variable, but after several re-crystallisations and using rapid heating to just below the melting-point, the sugar melted sharply at 134.5 - 135.5°C. This agreed quite well with the value 137-137.5°C given by Whistler and Smith (15).

The trisaccharide had an initial rotation $[\alpha]_D^{17}$ -15.7° (extrapolated to zero time) changing after 2 hours to -20.2° (equilibrium value)(C, 3.7 in water). Whistler and Smith report the values $[\alpha]_D^{25}$ -24.7° changing to -23.3°(C 1.29 in water) for β -1:4 mannotriose. They found that the sugar crystallises as the trihydrate. Analysis of the crystalline trisaccharide from Mannan A gave the following results:-

Found:	C, 39.04%,	H, 7.085%
$C_{18}H_{32}O_{16} \cdot 3H_2O$ requires	C, 38.7%,	H, 6.861%
$C_{18}H_{32}O_{16}$ requires	C, 42.84%	H, 6.393%

Preparation of a derivative of mannotriose

1. Whistler and Smith (15) have prepared a crystalline mannotriitol acetate. They reduced the trisaccharide by shaking the aqueous solution with hydrogen at 45 p.s.i. in the presence of a platinum catalyst. Using the same conditions it was found that the trisaccharide was not completely reduced after 9 days shaking.

2. The trisaccharide (100 mg.) was dissolved in water (2 ml.) and potassium borohydride solution (8 mg. in 0.1 ml.) was added. After 4 hours the solution was still slightly reducing as was shown by spotting a little on to a filter-paper and spraying with aniline oxalate solution containing a few percent of phosphoric acid, but the reaction was stopped by adding excess dilute acetic acid. The solution was de-ionised by treating with Amberlite 1R 120 resin and evaporating with methanol (twice). Examination of the residue by paper chromatography using ethyl acetate-pyridine-water as the solvent and silver nitrate/sodium hydroxide (106) as the spray reagent showed that degradation of the trisaccharide had occurred with the production of considerable

quantities of mannitol.

3. Mannotriose (300 mg.) dissolved in 6 ml. water was treated with potassium borohydride solution (100 mg. in 2 ml. water). Reduction was complete in 45 minutes. Excess borohydride was destroyed by adding dilute acetic acid and the solution evaporated to dryness.

The dried syrup was acetylated by heating with acetic anhydride (12 ml.) and fused sodium acetate (150 mg.) at a temperature just below the boiling-point for 30 minutes. The solution was cooled and poured into ice-water; the mixture was stirred until the brownish oil solidified. Yield 250 mg. After several re-crystallisations from ethanol/light petroleum (B.P. 80-100°C) and ethanol the acetate had $[\alpha]_D^{18} -24^\circ$ (c 2.0 in chloroform) and melted at 112.5 - 115°C. Whistler and Smith report the values $[\alpha]_D^{25} -21^\circ$ (c 1.18 in chloroform) and melting point 113.5-114°C for this compound

Melting-point of the mannotriose

A few seed crystals of the mannotriose were sent to Dr. L. Hough and he has reported (91) that the sugar melts at c. 164°C. The melting-point was found on a Kofler micro-melting apparatus. The melting-point of the tri-saccharide was therefore re-examined. It was found that a carefully re-crystallised sample melted at 166.5-169.5°C

on the hot-plate. The melting-point on rapid heating in a tube, under the conditions previously used, was the same as before, namely $134.5-135.5^{\circ}$. Slower heating either on the hot-plate or in a melting-point tube produces sintering at c. 136° and final melting at a temperature above 160°C . The lower melting-point thus appears to be that of the trihydrate. This was confirmed by slowly heating the crystalline trisaccharide in a sealed tube. Melting occurred at $127.5 - 129.5^{\circ}\text{C}$.

Comparison of powder photographs

The X-ray powder photograph of the trisaccharide has been compared by Professor R.L. Whistler with that of an authentic specimen of β -1;4 mannotriose and shown to be identical with it.

The trisaccharide is therefore O- β -D-mannopyranosyl-(1 \rightarrow 4)-O- β -D-mannopyranosyl-(1 \rightarrow 4)-D-mannopyranose. From its mutarotation it appears to be the α -anomer, the discrepancy in the values with those given by Whistler and Stein has not been explained.

Fraction 5 (1.8 g.) This fraction, eluted with 10% ethanol, contained mannotriose and two other sugars, one of which travelled slightly faster (R_M 0.29) and the other rather slower (R_M 0.15) than the trisaccharide on a paper chromatogram.

Their chromatographic mobilities suggested that these two sugars were also trisaccharides and they were designated Trisaccharide X and Trisaccharide Y respectively. The fraction also contained a considerable amount of mannobiose as well as a little monosaccharide and what appeared to be tetrasaccharide.

Separation of Trisaccharides

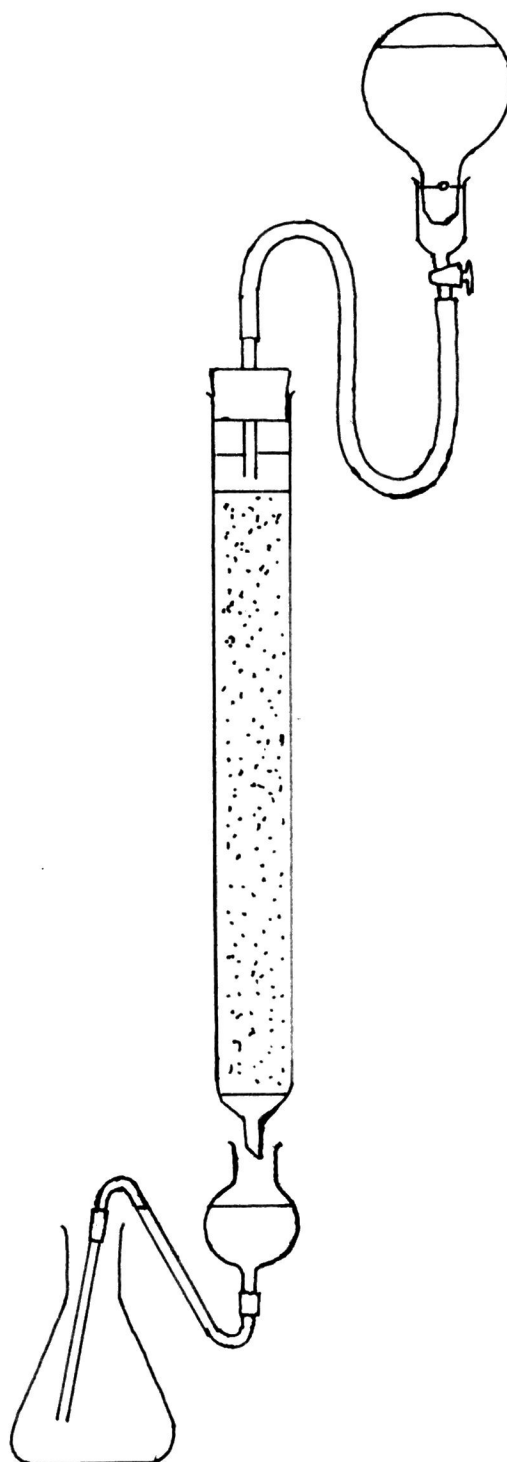
A cellulose column (22 x 400 mm.) was prepared, the cellulose powder being firmly tamped down with a thick glass rod. The column was washed with water (500 ml.) and solvent (500 ml.), the latter being ethyl acetate-pyridine-water. The pyridine was purified by refluxing with potassium permanganate and distilling. The sugars (1.27 g.) were dissolved in a small volume of water and the solution absorbed on discs of Whatman 3 MM paper, the discs having the same diameter as the column. The discs were air-dried and placed on top of the cellulose powder. In this way the difficulty of applying the sugars to the column was overcome. Elution was begun using a constant-level reservoir; the eluant was collected in 4-5 ml. fractions in test-tubes by means of the automatic turntable described by Hough, Jones and Wadman (92). Every tenth tube was evaporated to dryness at 80°C in a current of air, and the contents analysed

by paper chromatography. The tubes containing trisaccharides X and Y were combined and evaporated to dryness under reduced pressure. The dark brown syrups were dissolved in cold water and treated several times with charcoal and celite to remove wax and pyridine oxidation products. The remaining fractions were bulked. Recovery 94% Yields X = 205 mg.
Y = 95 mg.

Acetolysis of Mannan A (2)

Mannan A (55 g.) was treated with an acetolysis mixture (693 ml.) in the manner already described (p. 29) for 72 hours. The solution was poured into ice-water (2.5 litres), the pH adjusted to 3-4 and the mixture allowed to stand overnight as before. The supernatant liquid was decanted off and the solid washed with water several times by decantation, and dried by suction. The washings were added to the supernatant liquid which was then extracted with chloroform (3 x 800 ml.) The solid acetates were dissolved in chloroform, and the chloroform solutions combined, washed thoroughly with saturated sodium bicarbonate solution and water, and dried over anhydrous sodium sulphate. The chloroform was distilled off under reduced pressure and the glassy product allowed to stand in vacuo, over sodium hydroxide for 24 hours. Yield 90.7g.

CHARCOAL-CELITE COLUMN



Deacetylation of the mixed acetates

The acetates were dissolved in dry chloroform (250 ml.) and absolute methanol (500 ml.) was added. The solution was then deacetylated with N barium methoxide (75 ml.). Slightly less than the equivalent volume of dilute sulphuric acid (c.175 ml.) was added with vigorous shaking, followed by 175 ml. of water. The chloroform layer was separated off and the aqueous layer filtered through a sintered glass disc covered with a layer of Celite. The solution was evaporated to dryness and dried at 40°C in vacuo, over P_2O_5 . Yield 45.8g

Separation of oligosaccharides on a Charcoal-Celite column

A column 820 x 74 mm. was prepared from 1300 g. of a mixture of M. and B. decolourising charcoal and Johns-Manville Celite (Grade 545) in the ratio 2:1. The mixture was added in the form of a slurry and packed under light suction. The column was washed with 5-10 litres of water. The sugars were dissolved in water (200 ml) and absorbed onto the column under suction. Elution was begun with water and continued with aqueous ethanol. The ethanol concentration was increased progressively in steps of 0.5% or 1%. The column was fitted with a variable head device enabling the rate of flow to be controlled (see diagram). The effluent was collected in litre fractions. A sample (c.5 ml.) was

removed from each fraction and evaporated to dryness at 100° C in a current of air. The residue was analysed chromatographically using ethyl acetate-pyridine-water as the solvent. Fractions having the same composition were combined and evaporated to dryness in a large-scale evaporator. In this way 10 major fractions were obtained.

Fraction 1 (6.5g.)(0-4% ethanol) Monosaccharides; mannose; pentose; a little glucose, and galactose.

Fraction 2 (9.8g.)(4.5-6.5% ethanol) Mannobiose. The sugar crystallised readily without further purification.

Fraction 3 (128g.)(6.5-8% ethanol) Mixed disaccharides.

Fraction 4 (5.6g.)(9-10% ethanol) Mannotriose. This sugar also crystallised extremely readily.

Fraction 5 (1.84g.)(10.5-11.5% ethanol) Mixed trisaccharides.

Fraction 6 (2.1g.)(11.5-13% ethanol) This contained a single component which was tentatively identified as mannotetraose.

Fraction 7 (0.9g.)(13-14.5% ethanol) This fraction consisted principally of "mannotetraose", together with another sugar which moved faster than the tetrasaccharide on a paper chromatogram in ethyl acetate-pyridine-water. This sugar was thought to be also a tetrasaccharide.

Fraction 8 (0.9g.)(15-16%) This fraction appeared to contain a single sugar which moved only very slowly on a paper

chromatogram. It was considered to be mannopentaose.

Fraction 9 (0.8g.) This fraction contained a sugar which moved faster than "mannopentaose" on a chromatogram, though more slowly than the tetrasaccharides. Presumably this sugar was also a pentasaccharide. Considerable amounts of other oligosaccharides of lower D.P. were also present.

Fraction 10 (3g.) Elution was continued up to 20% ethanol but it was found to be impossible to follow the elution chromatographically. The column was therefore eluted with 6 litres of 25% aqueous ethanol.

Separation of the mixed disaccharides

The mixed disaccharides (fraction 3) were separated on a cellulose column, 25 x 400 mm, the solvent employed was ethyl acetate-1-propanol-water (16:10:5 v/v) which was allowed to stand for 24 hours before use. The sugars (1.2 g.) were applied to the column on paper discs in the way already described (p.56). Fractions (50 ml.) were collected by means of a Towers automatic fraction collector. A small sample (2-3 ml.) was withdrawn from each tube and the contents analysed chromatographically. The fractions containing Disaccharide C only were combined and evaporated to dryness. The residue was dissolved in a little cold

water and the solution centrifuged to remove waxy material from the cellulose. This procedure was repeated and the solution again evaporated to dryness.

Yield 180 mg.

Considerable overlapping occurred between manno-
biose and Disaccharide B; the fractions containing the
latter were therefore combined with a few which
contained a mixture of mannobiose and Disaccharide C
and evaporated to dryness. The mixture (275 mg.) was
then refractioned on a column of the same dimensions
as before. A further quantity (21 mg.) of chromato-
graphically-pure Disaccharide C was obtained together
with 98 mg. of slightly impure Disaccharide B. The
latter was separated from the last traces of manno-
biose by separation on Whatman 3 MM paper, using ethyl
acetate-1-propanol-water as solvent, and a chromato-
graphically-pure sample (58 mg.) obtained.

Attempted characterisation of disaccharide B

The sugar could not be crystallised by the methods
already outlined. The syrup had $[\alpha]_D^{17} + 49^\circ$ (c, 0.6,
in water). On hydrolysis the disaccharide gave mannose
only and will therefore be referred to as iso-
mannobiose.

Periodate oxidation - detection of formaldehyde. The disaccharide (c.6mg. in 1 ml.) was oxidised in the manner described by Reeves (93). The solution was treated with 0.3M sodium periodate solution (1 ml.) and 0.7N sodium bicarbonate solution (1 ml.) for 60 minutes. N Hydrochloric acid (3.5 ml.) was added followed by 1.2N sodium arsenite solution (c.2 ml.) and the mixture shaken until the yellow colour disappeared. Sodium acetate solution (M, 1 ml.) and 40mg. dimedone in 1 ml. of 95% ethanol were added and the solution treated at 100°C for 10 minutes and allowed to stand for one hour. A crystalline precipitate of the methylene-bis-dimedone compound was formed. M.P. and mixed M.P. 187-190°

Gentiobiose oxidised under the same conditions gave no precipitate with dimedone.

Preparation of the phenylosazone

Isomannobiose (10 mg.) was heated for 30 minutes on a boiling water-bath with 0.01 ml. each of phenylhydrazine and glacial acetic acid, 0.25 ml. water and a drop of saturated sodium bisulphite solution. On cooling an orange syrup was obtained which was warmed with water (1.5 ml.) and the mixture allowed to cool. The osazone was precipitated in a crystalline form and was re-crystallised

from boiling water to give spherulitic clusters of needles.

M.P. 200-201°C.

Oxidation with lead tetraacetate

Isomannobiose was oxidised with lead tetraacetate using the method of Perlin (80). The disaccharide (1 mg.) was dissolved in 0.01 ml. water and the solution diluted to 0.1 ml. with glacial acetic acid. Lead tetraacetate (5 mg.) in 0.4 ml. of glacial acetic acid was added. After two hours excess oxalic acid was added, and the solution diluted with water and heated on the water-bath with 1R 120 resin. No pentose could be detected in the hydrolysate, though a spot corresponding to a tetrose was present on the paper chromatogram. Under the same conditions laminaribiose gave arabinose.

Rotation in methanolic hydrogen chloride

A saturated solution of isomannobiose in methanol had a rotation of 0.15° , measured in a 2 dm. tube. To 10 ml. of this solution, 5 ml. of 2% methanolic hydrogen chloride were added. The rotation fell to the expected value, 0.10° (owing to the dilution of the solution) but there was no further change after standing for six hours.

Attempted characterisation of disaccharide C

The sugar was obtained as a syrup which could not be crystallised. The syrup had $[\alpha]_D + 5.5^\circ$ (c, 3.5 in water). On hydrolysis the disaccharide gave mannose and glucose in equal proportions.

Bromine water oxidation and hydrolysis

The disaccharide (1 mg.) was treated with bromine water (0.75 ml.) for 24 hours at room temperature. The bromine was removed by aeration and the solution neutralised with silver carbonate and filtered. The oxidised disaccharide was then hydrolysed by adding dilute sulphuric acid and heating the solution at 100°C for 3 hours. A chromatogram of the hydrolysate showed that only mannose was present.

Preparation of the phenylosazone and comparison with mannobiosazone

The osazone was prepared in the same way as isomannobiose. The osazone did not crystallise readily at first but after the solution had been shaken with ether to remove traces of tarry material it crystallised in the form of bundles of needles, or on slow cooling, leaf-shaped crystals. M.P. $149-152^\circ\text{C}$

Mannobiosazone, prepared in the same way, crystallised in the form of spherulitic aggregates of needles which melted at $203-206^\circ\text{C}$.

X-ray powder photographs were taken of the two compounds and compared. It was found that there were significant differences in the high-order reflections. The strong low-order reflections appear to be very similar in a number of different osazones.

Periodate oxidation - detection of formaldehyde

The disaccharide was oxidised with sodium metaperiodate in bicarbonate buffer in the manner already described (p.62). On heating with dimedone solution a crystalline precipitate was formed of the methylene-bis-dimedone compound. M.P. and Mixed M.P. 187-190°C.

Lead tetraacetate oxidation

The mannosyl glucose was oxidised in the same way as isomannobiose and the product hydrolysed and examined chromatographically. No pentose was detected, although tetrose was present.

Separation of Trisaccharides X and Y

The mixture of Trisaccharides (fraction 5, 1.8 g.) was separated on a cellulose column using ethyl acetate-isopropanol-water as solvent. Slight overlapping occurred, but pure samples of Trisaccharides X (320 mg.) and Y (116 mg.) were obtained.

Attempted characterisation of Trisaccharide X

The trisaccharide was crystallised by adding ethanol to the hot aqueous solution and allowing the mixture to cool slowly to c. 35°C. The solution was kept at this temperature overnight. The sugar crystallised in the form of tiny spherulites. By allowing the

solution to evaporate slowly at room temperature a second crop of crystals was obtained in the form of spherulitic clusters of needles. The trisaccharide melted at 224-225°C and had $[\alpha]_D^{18} + 43.2^\circ$ (c 2.1 in water) after 5 minutes changing after 1 hour to $+ 39.8^\circ$.

Hydrolysis of the Trisaccharide

Total hydrolysis of the trisaccharide (3 hours, N sulphuric acid at 100°C) yielded only mannose. Partial hydrolysis by heating for 1 hour with N sulphuric acid gave mannose and a sugar which had the same chromatographic mobility as mannobiose. Accordingly, samples of the trisaccharide were partially hydrolysed by heating for 1 hour at 100°C with (1) 0.25 N sulphuric acid, and (2) 0.1 N sulphuric acid. In both cases paper chromatograms of the hydrolysate showed that, in addition to mannobiose, a second disaccharide was present which was chromatographically identical with isomannobiose.

Borohydride reduction and partial hydrolysis of isomannotriose

The trisaccharide (1 mg. in 0.1 ml.) was treated with the same weight of potassium borohydride dissolved in 0.1 ml. of water. After 1 hour the excess borohydride was destroyed with dilute acetic acid and the solution de-ionised in the

manner already described (p.66). The residue was partially hydrolysed as above and the hydrolysate examined on a paper chromatogram. Only mannose and isomannobiose were present.

Attempted characterisation of Trisaccharide Y

This sugar could not be crystallised. The syrup had $[\alpha]_D^{17} - 7.0^\circ$ (c, 4.1 in water).

Hydrolysis of Trisaccharide Y

On complete hydrolysis the trisaccharide gave mannose and glucose in the ratio 2:1 (by inspection). Partial hydrolysis under mild conditions (0.1N sulphuric acid for 1 hour) gave mannose, glucose, mannobiose, and mannosyl glucose. Under more stringent conditions (N sulphuric acid for 1 hour) no mannosyl glucose could be detected.

Borohydride reduction and partial hydrolysis of Trisaccharide Y

The trisaccharide (1 mg.) was reduced with potassium borohydride and the product partially hydrolysed in the same way as for isomannotriose. The products were mannose and mannobiose.

Investigation of fractions 6 and 8

Fraction 6 was crystallised in the form of micro spherulites

from hot ethanol/water, in the usual way. After several recrystallisations the sugar melted sharply at 231.5 - 232°C. and had $[\alpha]_D^{20}$ -31.6° after 5 minutes, changing to -28.7° after 50 minutes (c , 0.9 in water).

Total hydrolysis of the sugar (4 hours in N sulphuric acid at 100°C.) gave only mannose. Partial acid hydrolysis (1 hour in 0.1 N sulphuric acid) gave rise to the homologous series:- mannose, mannobiose, mannotriose.

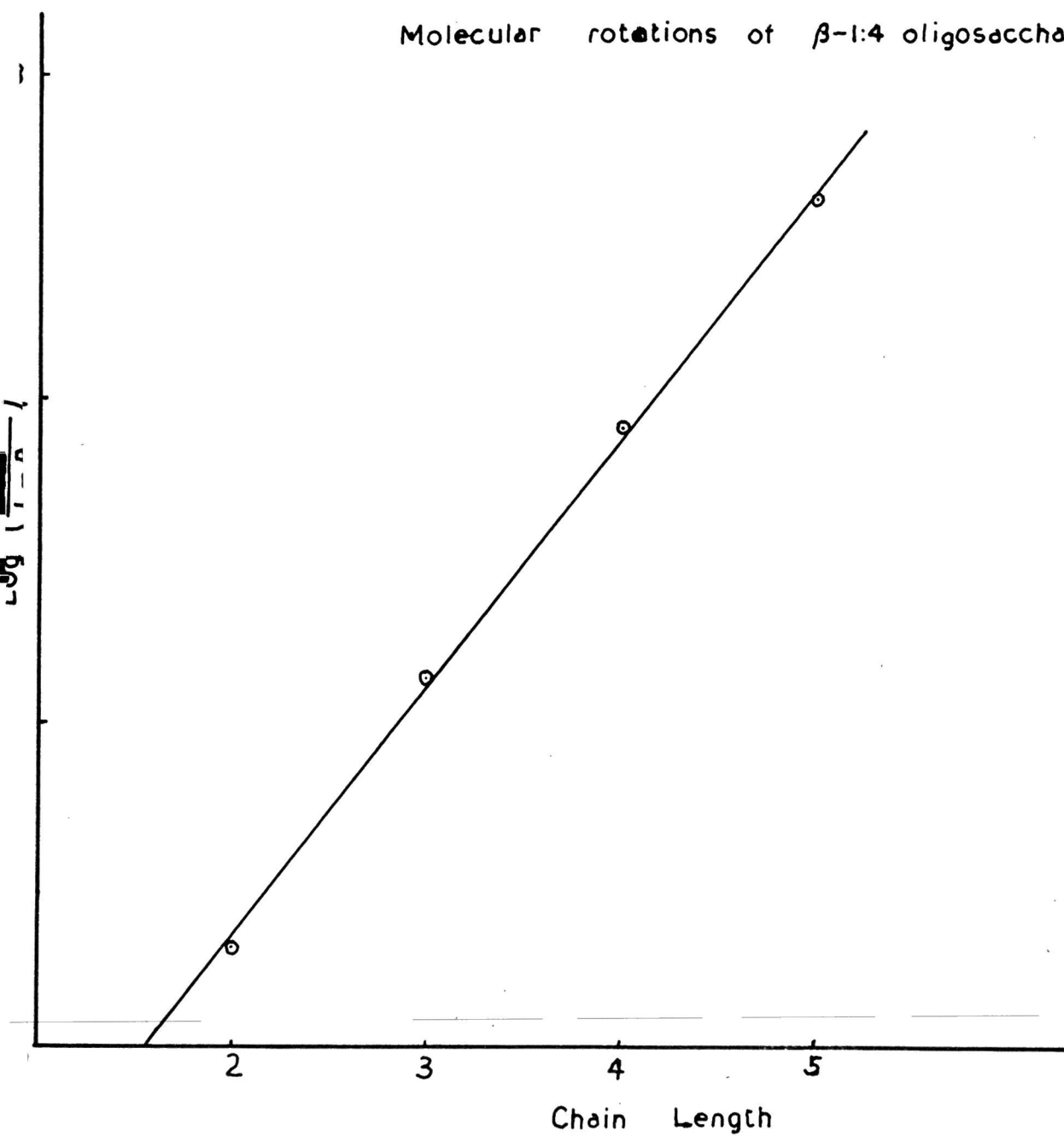
Fraction 8 was also crystallised from ethanol/water. The crystals turned brown but did not melt below 280°C. The sugar had $[\alpha]_D^{20}$ - 30.2° (c , 2.1 in water), any mutarotation was too small to be observed. On complete hydrolysis the oligosaccharide gave only mannose. The partial hydrolysis products were mannose, mannobiose, mannotriose, and (presumably) mannotetraose.

A portion of Fraction 9 was refractionated chromatographically on Whatman 3MM paper using ethyl acetate-pyridine-water as solvent, and a sample (20 - 30 mg.) of the chromatographically pure pentasaccharide was obtained. On complete hydrolysis it gave mannose, and on partial hydrolysis: mannose, mannobiose, isomannobiose, mannotriose, isomannotriose, mannotetraose, and "isomannotetraose".

A portion of Fraction 7 was separated in the same way and a sample of the tetraose, slightly contaminated with mannotetraose obtained. It gave only mannose on complete hydrolysis, and mannose, mannobiose, isomannobiose, mannotriose, and isomannotriose on partial hydrolysis.

GRAPH No.4.

Molecular rotations of β -1:4 oligosaccharides



MANNAN B

Extraction of Mannan B

Mannan B was extracted from the residues of the Mannan A extractions by a modification of the method of Aspinall, Hirst, Percival, and Williamson (70). The residues, which had been washed free of alkali and dried, were extracted in 50g batches. The material was allowed to swell in concentrated ammonia (94). Sucrose was added to minimise oxidation (95), and the mixture was shaken with 1.5 - 2 litres of cuprammonium hydroxide solution prepared by the method of Carver (96) for 16 hours. In order to minimise oxidation of the mannan the extraction bottles were almost completely filled with solution. The mixture was filtered rapidly through a glass wool plug, and 2N sodium hydroxide solution was added slowly, with stirring, until the solution was 0.2N with respect to sodium hydroxide. The bulky gelatinous precipitate which formed was centrifuged off, stirred with water, and acidified with glacial acetic acid. Some precipitation occurred at this stage; the addition of an equal volume of methylated spirits produced a further copious precipitation. Each batch of material was extracted three times; in all 230g. were extracted.

The crude mannan mixture was shaken for several hours with 7% potassium hydroxide solution(2 l.) and the insoluble

material separated at the centrifuge. The solution, on acidification and the addition of an equal volume of methylated spirits, gave a considerable precipitate of (presumably) Mannan A. The residue was washed with 7% potassium hydroxide solution and water, and dried with ethanol and ether. On hydrolysis it gave mannose and glucose in the ratio 2:1 approximately.

Final purification of Mannan B was effected by extraction with anhydrous formic acid, a method suggested by Dr. J.K.N.Jones (97) and used by Aspinall, Hirst, Percival and Williamson (70). The crude mannan was shaken for several hours with anhydrous formic acid (400 ml.) and the mixture centrifuged. The residue was extracted with a further 400 ml. formic acid and the combined extracts were poured into an equal volume of ethanol. The precipitate was washed with ethanol and dried with ether. Yield 15.4 g.

On hydrolysis (14 hours with formic acid at 100°C) the product gave mannose with only traces of glucose and galactose. The mannan had $[\alpha]_D^{18} -26^\circ$ (c, 1.0, in anhydrous formic acid) which is the same value as that given by the above-mentioned authors (70). It was concluded therefore that the product was pure Mannan B.

Prepared in the manner described Mannan B was a horny, rather intractable material. In order to convert it to a more readily soluble form the polysaccharide was allowed to swell in water and the slurry freeze-dried. Mannan B was left in a bulky, highly porous condition.

Graded Acetolysis of Mannan B

Preliminary small-scale studies of the acetolysis of Mannan B under similar conditions to those employed for Mannan A, showed that it was much less readily degraded than the latter polysaccharide but that reasonable yields of oligosaccharides could be obtained after a reaction period of 96 hours. The mannan (13 g.) was added to a mixture of acetic anhydride (80 ml.), glacial acetic acid (80 ml.), and concentrated sulphuric acid (8 ml.), in the cold. After standing for 96 hours, the mixture was poured into 750 ml. of ice-water and the pH adjusted to 3.4 with sodium bicarbonate. The mixture was allowed to stand overnight and the solid acetates were filtered off. The solution was extracted three times with chloroform (500 ml. portions) and the solid material was dissolved as far as possible in the combined extracts. The chloroform solution was filtered, washed with saturated sodium bicarbonate solution, and water, and dried over anhydrous sodium sulphate:

it was then concentrated under reduced pressure and the resulting syrup allowed to stand for 24 hours in vacuo over sodium hydroxide. Yield 14.6 g.

Deacetylation of Mannan B acetolysate

Some of the acetate mixture was lost in an accident but the remainder (10.1 g.) was dissolved as far as possible in 150 ml. of anhydrous methanol and deacetylated with barium methoxide (15 ml., 0.9N) in the manner described for Mannan A (pp. 42). The solution of free sugars obtained was freeze-dried since it frothed very violently when evaporated under reduced pressure. Yield 4.8 g.

Separation of oligosaccharides on a Charcoal-Celite Column

A column (45 x 450 mm.) was prepared, from equal quantities of B.D.H. activated charcoal and Johns-Manville Celite, grade 545, in the manner described on p. 58. The column was washed with 5-10 litres of water and the sugars, dissolved in water (150 ml.) absorbed on to it. Elution was begun with water (1 litre) and continued with aqueous ethanol, the concentration being increased in steps of 1%. The effluent was collected in 250 ml. fractions. Samples (0.5 ml.) from each fraction were evaporated at 100°C in a current of air and analysed by paper chromatography using ethyl acetate-pyridine-water as solvent.

The fractions were combined into seven main fractions.

The recovery was 94%.

Fraction 1 (425 mg.)(1-2% ethanol) Contained only monosaccharides, mannose, pentose, and traces of glucose and galactose.

Fraction 2 (920 mg.)(4-6% ethanol). This fraction consisted of a single, chromatographically pure sugar, which travelled at the same rate on a paper chromatogram as mannobiose. The disaccharide crystallised readily from ethanol/water using the technique described on p. 49 . Its melting point was 203-206°C undepressed on admixture with a sample of mannobiose and it gave an X-ray powder photograph identical with the latter sugar. The fraction consisted therefore of 4-O- β -D-mannopyranosyl—D-mannopyranose.

Fraction 3 (84 mg.)(7-8% ethanol) Was a mixture of mannobiose with two other disaccharides which were chromatographically identical with disaccharides B and C from Mannan A. Monosaccharides and a little trisaccharide were also present.

Fraction 4 (600 mg.)(9% ethanol) gave only one spot on a paper chromatogram which moved at the same rate as mannotriose. The sugar crystallised readily from ethanol/water; after recrystallisation it displayed the same melting-point behaviour as mannotriose, its melting-point on slow heating being 163-167°C

undepressed on admixture with mannotriose. The X-ray powder photographs of the two sugars were also identical. Hence the trisaccharide was $\text{O}-\beta\text{-D-mannopyranosyl}-(1\rightarrow4)-\text{O}-\beta\text{-D-mannopyranosyl}-(1\rightarrow4)-\text{O}-\text{D-mannopyranose}$.

Fraction 5 (166 mg.)(10-11% ethanol) was a mixture of mannotriose and two other trisaccharides which appeared, from their chromatographic mobilities, to be the same as trisaccharide X and Y from Mannan A. The fraction also contained some tetrasaccharide and mono- and disaccharide impurities.

Fraction 6 (256 mg.)(12% ethanol) consisted of a single sugar which had the same rate of movement on a paper chromatogram as the mannotetraose isolated from Mannan A. The sugar crystallised readily using the same technique as for mannotriose. It melted at $231.5-232^{\circ}\text{C}$ (M.P.tube), the melting point being undepressed on admixture with mannotetraose. The sugars also gave identical X-ray powder photographs.

Fraction 7 (1.410 g.) The fractions eluted with 13% and 14% aqueous ethanol consisted of mixtures of oligosaccharides. The column was therefore eluted with 25% aqueous ethanol (1 litre).

Separation of mixed oligosaccharide fractions from Mannan B

Although the mixed fractions (3 and 5) would obviously yield only small amounts of the anomalous sugars, a

separation was carried out in order to discover if a similar method could be applied to the much larger amounts of these sugars obtained from Mannan A.

A cellulose column (14 x 400 mm) was prepared and washed with water (200 ml.) and solvent (200 ml.). The solvent employed was ethyl acetate-isopropanol-water (16:10:5 v/v) which had been allowed to equilibrate for 24 hours. The oligosaccharide mixture (250 mg.) was dissolved in a little water and the solution taken up on 10 discs of Whatman 3 MM paper. After air-drying, the discs were placed in position and elution commenced. The effluent was collected in 2 ml. fractions in test-tubes, automatically, the contents of every tenth tube being examined by paper chromatography in the usual way. Some overlap was noted between Disaccharide B and mannobiose, and between Trisaccharide X and mannotriose, but small amounts of each of the four anomalous sugars were obtained. The recovery was 81%.

<u>Results</u>	<u>Sugar</u>	<u>Yield</u>
	Disaccharide B	8 mg.
	Disaccharide C	14 mg.
	Trisaccharide X	28 mg.
	Trisaccharide Y	20 mg.
	Remainder	134 mg.

GLUCOMANNAN FROM SITKA SPRUCE

DISCUSSION

A glucomannan extracted by Dr. R. A. Laidlaw from the wood of Sitka Spruce has been the subject of a preliminary examination. The glucomannan was extracted with 10% sodium hydroxide from sawdust which had been delignified twice with sodium chlorite and acetic acid. Three different samples were obtained at different times.

The first sample gave mannose and glucose on hydrolysis (formic acid) in the ratio 1:1 approximately. Attempts to obtain a pure mannan by extracting the crude material with formic acid, and by fractionating the acetate, were unsuccessful. The polysaccharide, or polysaccharide mixture, was therefore methylated, first with the Haworth reagents, sodium hydroxide and dimethyl sulphate, and then with silver oxide and methyl iodide. The methoxyl content remained constant at 42% in spite of repeated treatments with the latter reagent. No fractionation could be achieved by extraction with mixtures of light petroleum and chloroform of increasing chloroform content.

The methylated product which had $[\alpha]_D^{20} -9^\circ$ was hydrolysed with anhydrous formic acid and dilute hydrochloric acid, and the hydrolysate fractionated on a cellulose column. The principal fraction which had the same chromatographic mobility as the 2:3:6-tri-O-methyl derivatives of D-glucose and D-mannose

crystallised spontaneously on standing. The substance was recrystallised and identified from its melting point, mixed melting-point, mutarotation, and change of rotation in dilute methanolic hydrogen chloride, as 2:3:6-tri-O-methyl-D-glucose. A fraction which had a chromatographic mobility corresponding to a tetra-O-methyl hexose gave only glucose on demethylation. A third fraction, which contained one or more di-O-methyl hexoses, was not investigated. Assuming a linear structure for the polysaccharide, the amount of tetra-O-methyl-D-glucose isolated corresponds to a D.P. of about 80, and it is clear that the product was essentially a methylated degraded cellulose. Clearly the crude polysaccharide must have been a mixture of short-chain cellulose with a mannan or glucomannan. It appeared likely that the mannose-containing material had been lost during the isolation of the methylated product from the Haworth methylation.

A second sample of the "glucomannan" was methylated with sodium hydroxide and dimethyl sulphate and attempts were made to isolate a mannose-containing fraction from the solution. Since no material could be extracted with chloroform this could only be achieved by dialysis, and the partially methylated fraction isolated gave only glucose on

hydrolysis and demethylation.

The third sample supplied by Dr. Laidlaw had a mannose:glucose ratio of 2-2.5:1, accurate values could not be obtained because of the difficulty of obtaining a complete separation of mannose and glucose on paper chromatograms. The glucomannan had $[\alpha]_D^{25} -33^{\circ}$ (c, 1.1 in 2N sodium hydroxide).

The polysaccharide was subjected to oxidation by the periodate ion, and measurements were made of the periodate uptake and the amount of formic acid released. The uptake found (by extrapolation) was 1.14 moles of periodate, per mole of anhydrohexose, suggesting that the polysaccharide is either 1:4 or (less probably) 1:2-linked (theoretical uptake 1 mole/mole for an infinite polymer). The amount of formic acid released increased steadily throughout the oxidation and although there was a decrease in the rate of evolution after one mole of formic acid had been produced for every 15 moles of anhydrohexose, it was impossible to draw any conclusions regarding the chain-length of the polysaccharide from this experiment.

The glucomannan was methylated by treating the polysaccharide with thallous hydroxide and refluxing the thallium derivative with methyl iodide (81). After three methylations

a good yield of a chloroform-soluble product was obtained which was further methylated with the Purdie reagents, silver oxide and methyl iodide. The material was purified by pouring the chloroform solution into a large excess of light petroleum. A portion of the material was soluble in the mixture but was not further investigated. The precipitate, which was considered to be methylated glucomannan, was re-methylated with the Purdie reagents, but the methoxyl content remained unchanged at 42%.

The methylated glucomannan which had $[\alpha]_D^{18} - 13^\circ$ (C, 2.5 in chloroform) was hydrolysed successively with anhydrous formic acid and dilute hydrochloric acid. The hydrolysis products were separated on a cellulose column, and four fractions were obtained. The first fraction, obtained as a syrup, had the same chromatographic mobility as the 2:3:4:6-tetra-O-methyl derivatives of D-mannose and D-glucose. The syrup had $[\alpha]_D^{18} + 8^\circ$ (C, 1.7 in water) suggesting that it consisted almost entirely of 2:3:4:6-tetra-O-methylmannose ($[\alpha]_D^{25} + 2^\circ$).

The second fraction gave a single spot on a paper chromatogram which had the same R_M value as the 2:3:6-tri-O-methyl derivatives of D-mannose and D-glucose. The syrup had $[\alpha]_D^{18} + 10.8^\circ$ (C, 3.5 in water) suggesting that it

was a mixture of these two compounds. The two methylated sugars were separated by selective furanoside formation; when a mixture of the sugars is treated with cold dilute methanolic hydrogen chloride, the methyl furanoside of 2:3:6-tri-O-methyl-D-glucose is formed preferentially. The reaction can be followed by the change in rotation of the solution (22). The glycoside was separated from the unchanged 2:3:6-tri-O-methyl-D-mannose on a cellulose column, and hydrolysed. The two methylated sugars were characterised as the crystalline di-p-nitrobenzoyl esters. The ratio of 2:3:6-tri-O-methyl-glucose to 2:3:6-tri-O-methyl mannose was estimated by comparing the optical rotation of the mixture ($+10.8^\circ$) with those of 2:3:6-tri-O-methyl-D-glucose ($+70.5^\circ$) and 2:3:6-tri-O-methyl-D-mannose (-10° ; various other figures of this order have been quoted). The ratio was determined by consideration of the change in rotation of the mixture in dilute methanolic hydrogen chloride; the change was 26° compared with 103° for pure 2:3:6-tri-O-methyl glucose and zero for 2:3:6-tri-O-methyl mannose. Thus the two methods give virtually the same value (somewhat fortuitously perhaps) for the ratio, namely 3:1.

A small-scale acetylsis has been carried out on the

glucomannan and two disaccharide fractions obtained by chromatographic separation of the products on thick paper. The main fraction, which appeared on a paper chromatogram to be a single sugar, was shown by ionophoretic analysis to be a mixture of mannobiose with a little cellobiose. The other fraction gave a single spot which travelled both on chromatograms and ionophoretograms, at the same rate as the mannosyl glucose from ivory nut mannans. Since it gave mannose and glucose on hydrolysis, and the sugar alcohol, prepared by borohydride reduction, gave mannose as the only reducing sugar on hydrolysis, it appeared to be identical with the latter sugar. Traces of two other disaccharides could also be detected.

The isolation, in fair yield, of a disaccharide giving both mannose and glucose on hydrolysis, from the partial hydrolysis products of the polysaccharide show that it must be a glucomannan, though it may of course be contaminated with glucan. The low optical rotation of the glucomannan suggests that most of the linkages are of the β -glycosidic type, while the results of the methylation studies and the periodate oxidation studies indicate that the sugar residues are linked in the 4-position. The presence of mannobiose and cellobiose in the partial hydrolysis products also

supports this view.

Since methylation was incomplete it is difficult to assess the significance of the di-O-methyl fraction isolated. It seems probable that the polysaccharide consists of chains of β -1:4-linked mannose and glucose residues, though a small degree of branching may occur.

GLUCOMANNAN FROM SITKA SPRUCE

A glucomannan has been obtained by Dr. R. A. Laidlaw from the wood of Sitka Spruce. The extraction procedure employed may be summarised as follows. Air-dried sawdust was extracted with aqueous ethanol and benzene. The extractive-free material was extracted with cold and hot water, when a small amount of carbohydrate was obtained. The sawdust was delignified by treatment with sodium chlorite and acetic acid. The residue, which still contained 5.5% lignin, was extracted with 4% sodium hydroxide. The extract gave on hydrolysis, galactose, glucose, mannose, arabinose, and xylose. Exhaustive extraction with 24% potassium hydroxide yielded a product which gave the same sugars on hydrolysis. The residual material was again delignified and exhaustively extracted with 10% sodium hydroxide. The glucomannan was precipitated as the copper complex by the addition of Fehling's solution to this extract. The complex was decomposed with acetic acid, and the gluco-mannan twice purified by dissolving in 8% sodium hydroxide and precipitating the copper complex. The product was dried over calcium chloride and gave mannose and glucose in the ratio 1:1 (approx.) on hydrolysis. The yield was 10g.

Attempted purification of the crude polysaccharide

1. The polysaccharide (1g.) was shaken for 24 hours with

anhydrous formic acid (50 ml.) and the mixture centrifuged. The extract was poured into ethanol and a considerable precipitate formed (605 mg.). The residue (305 mg.) was washed with a little formic acid and samples of the soluble and insoluble material were hydrolysed by heating for 16 hours at 100°C with anhydrous formic acid. The hydrolysates were examined on a paper chromatogram and appeared to be virtually identical.

2. The polysaccharide was acetylated by the method of Carson and MacLay (98). The material (200mg.) was heated in formamide (5 ml.) at 60-65°C for 2 hours, pyridine (5 ml.) was added and the mixture cooled to room temperature. Acetic anhydride (1.5 ml.) was added and the mixture shaken for 3 days. A portion of the material did not dissolve, the mixture was therefore centrifuged and the insoluble residue washed with pyridine and water. The centrifugate was poured into ice-water and the precipitate washed by decantation and at the pump. The two fractions were dried over potassium hydroxide.

Yields: Soluble fraction 71 mg.

Insoluble fraction 139 mg.

The fractions were deacetylated by the Zemplén method with sodium methoxide (99) and the products hydrolysed by heating for 14 hours in anhydrous formic acid. The hydrolysates

were examined by paper chromatography and showed only slight differences, the soluble acetate fraction appeared to give slightly more mannose.

Methylation of Spruce Mannan

The polysaccharide (7.4 g.) was methylated as follows. The material was stirred for several hours with 40% sodium hydroxide (200 ml.) in a three-necked flask. The air in the flask was displaced by oxygen-free nitrogen and the whole methylation was conducted under a slow stream of nitrogen. The flask was cooled in water and dimethyl sulphate (100 ml.) was added dropwise over a period of 4 hours with vigorous stirring. Four more additions of reagents were carried out in the same way. The flask was heated on the steam-bath for 30 minutes to destroy sodium methyl sulphate and the solution brought to pH 8 with glacial acetic acid. The hot supernatant liquid was decanted from the precipitated polysaccharide, and the slurry cooled and dialysed in a cellophane bag against running water for 24 hours. The mixture was then evaporated to dryness under reduced pressure. The solid was dispersed in water (150 ml.), treated five times with sodium hydroxide and dimethyl sulphate and isolated in the same way as before. The crude material was extracted repeatedly with hot chloroform

and the extracts combined, dried over anhydrous sodium sulphate, and evaporated to dryness. Yield: 5 g.

OMe: 38.7%

The glucomannan was remethylated by seven additions of 40% sodium hydroxide (90 ml.) and dimethyl sulphate (50 ml.)

Yield: 3.5g.

OMe: 41.6%

The polysaccharide was further methylated by dissolving in methyl iodide (50 ml.) and boiling the solution under reflux with silver oxide (2g.). Silver oxide (23 g.) was added at intervals over six hours. The mixture was re-fluxed overnight, the methyl iodide distilled off, and the solid extracted repeatedly with chloroform.

Yield: 3.1g.

OMe: 42%

An attempt to fractionate the material by extraction with mixtures of chloroform and light petroleum was unsuccessful. Two further methylations with silver oxide and methyl iodide were carried out and the product purified by pouring into a large excess of light petroleum. The solid was separated at the centrifuge, washed with light petroleum and the solvent removed under reduced pressure. The methoxyl content was unchanged at 42%. The methylated

product had $[\alpha]_D^{20} -9.4^\circ$ (c, 3.5 in chloroform).

Yield: 2.1g.

Hydrolysis of the methylated polysaccharide

A portion of the methylated polysaccharide (1.7g.) was hydrolysed by heating with anhydrous formic acid (40 ml.) for 5 hours at 100°C . The formic acid was distilled off under reduced pressure, the last traces being removed as the azeotrope by the addition of water and repeated redistillation. The dark brown syrup was then heated on a boiling water-bath for 3 hours with N hydrochloric acid to hydrolyse formyl esters. The solution was neutralised with silver carbonate and filtered; hydrogen sulphide was passed, the solution filtered again, and then concentrated to a syrup under reduced pressure and dried in vacuo over phosphorus pentoxide. The syrup tended to crystallise on standing. Paper chromatographic analysis showed that it consisted almost entirely of a sugar which had the same chromatographic mobility as 2:3:6tri-O-methyl mannose and 2:3:6 tri-O-methyl glucose. Yield: 1.61g.

Separation of the hydrolysis products on a cellulose column

A cellulose column (27 x 600 mm.) was prepared in the manner described by Hough, Jones and Wadman (92) and washed with water, n-butanol saturated with water, and

solvent. The solvent was a mixture of n-butanol, purified by distillation over sodium hydroxide, and light petroleum, (B.P. 100-120°C) purified by treatment with concentrated sulphuric acid, in the ratio 40:60 (v/v), saturated with water. The fractions (4-5 ml.) were collected in test-tubes and examined chromatographically in the manner already described (p. 56) using butanol-ethanol-water as solvent. The contents of the test-tubes with the same sugar were combined and evaporated to dryness under reduced pressure. The residues were taken up in a little cold water and treated with charcoal and celite. The solutions were filtered through Whatman No. 542 filter-paper and evaporated to dryness. In this way three fractions were obtained. The recovery was 89%.

Fraction 1 (Tubes 91-400)(18 mg.) This sugar gave a pink spot on a paper chromatogram sprayed with aniline oxalate, and had the same chromatographic mobility as the 2:3:4:6 tetramethyl derivatives of mannose and glucose. A sample (5 mg.) was demethylated, by the method of Hough, Jones, and Wadman (100), by heating for 10 minutes with 48% hydrobromic acid (1 ml.) at 100°C. The solution was cooled, diluted, neutralised with silver carbonate,

concentrated under reduced pressure, and examined on a paper chromatogram. Glucose, with perhaps a trace of mannose was present.

Fraction 2 (Tubes 400-800)(1.325g.) This fraction crystallised spontaneously in the form of needles, on standing, and when demethylated gave only glucose. A sample of the crystals was freed from traces of syrup by standing on porous tile in a vacuum dessiccator for 2 weeks. After two recrystallisations from ether/light petroleum (B.P. 60-80°C) the substance melted at 115-117°C undepressed on admixture with an authentic sample of 2:3:6 tri-O-methyl-D-glucose and had $[\alpha]_D^{17} +100.5^\circ$ (initial value) changing after six hours to $+70.6^\circ$.

The trimethyl sugar had an optical rotation in cold dilute methanolic hydrogen chloride of $+58.5^\circ$ changing after 28 hours to -36° , showing that there is a free hydroxyl group on C₄ and in agreement with the values $[\alpha]_D +70.5^\circ \rightarrow -33^\circ$ given by Irvine and Hirst (101). The fraction therefore consisted on 2:3:6 tri-O-methyl glucose.

Fraction 3 (Tubes 1120-1420)(93 mg.) This fraction contained one or more dimethyl hexoses and was not

investigated further.

Methylation of Spruce Mannan II

A fresh sample (4g.) of the mannan was methylated with seven additions of dimethyl sulphate (40 ml.) and 30% sodium hydroxide (110 ml.) in the manner already described. The mixture was heated for 15 minutes on the water-bath and neutralised with glacial acetic acid. The supernatant liquid was decanted off, cooled, and extracted with chloroform (4 x 250 ml.). The extract yielded a negligibly small amount of material. A portion of the precipitated solid was isolated, hydrolysed with formic acid and demethylated. Chromatographic analysis showed that only glucose was produced. The supernatant liquid was dialysed against running water for 48 hours and evaporated to dryness. The product also yielded glucose on hydrolysis and demethylation.

SPRUCE GLUCOMANNAN (2)

A third sample of Spruce glucomannan was supplied by the Forest Products Research Laboratory. The material was extracted as before, but was not purified by copper-complexing as this appeared to reduce the yield. As supplied the polysaccharide was very insoluble; it was therefore allowed to swell in water and freeze-dried. Hydrolysis with

formic acid (14 hours) showed that the main products were mannose and glucose in the ratio 2:1 (approximately), together with a trace of xylose. It was observed however that a certain amount of insoluble material was left after the hydrolysis treatment.

Measurement of percentage hydrolysis

A known weight of the glucomannan (29.46 mg.) was added to 70% sulphuric acid (1 ml.) at 0°C and allowed to stand in the cold for several hours with occasional shaking until it dissolved. The solution was then allowed to stand in the cold for a further 45 hours and diluted till it was normal with respect to sulphuric acid. The solution was heated for 3 hours at 100°C. The solution was cooled and a weighed sample (9.48g.) of xylose was added as a reference sugar. After neutralisation with barium carbonate the solution was concentrated to a small volume and spotted on to a paper chromatogram marked with side-strips. The paper was run for 18 hours in ethyl acetate-pyridine-water and air-dried. The side-strips were cut off, sprayed with aniline oxalate and the portions containing the hydrolysis products (mannose and glucose) and xylose were cut off. The strips were freed from the last traces of solvent by

suspending them over water in a dessiccator which was continuously evacuated for one hour. The sugars were eluted with cold water and estimated by periodate oxidation, using the method of Hirst and Jones (102). The formic acid released was determined by titration with carbonate-free sodium hydroxide (0.01N) using carbon dioxide-free water for rinsing and methyl red as indicator. Blanks were run at the same time.

Results

The mannose and glucose gave formic acid on oxidation which required 4.01 ml. of 0.01060N sodium hydroxide solution for neutralisation, while that from xylose required 3.09 ml. Since 1 mole of hexose gives 5 moles of formic acid and 1 mole of pentose gives 4 moles of formic acid, the above figures correspond to an apparent degree of hydrolysis of 40.1%.

Measurement of the ash content of the crude material showed that it was 28.3%.

Purification of the glucomannan

The material (14 g.) was macerated with water (50 ml.) and an equal volume of acetone was added to the suspension. The precipitate was separated and washed at the centrifuge with acetone/water (1:1 v/v). During the later washings the

material showed a tendency to become colloidal. The mannan slurry was transferred to a round-bottomed flask, the acetone distilled off, and the residue freeze-dried. Yield: 6.8 g.

A small amount (100 mg.) of water-soluble material was recovered from the washings by pouring into acetone. It gave mannose, galactose and xylose on hydrolysis with N sulphuric acid. The (sulphated) ash content of the purified glucomannan was 2.5%.

Measurement of the percentage hydrolysis of the purified glucomannan

The percentage hydrolysis was measured in the same way as before, using 39.55 mg. of polysaccharide and rhamnose (22.05 mg.). The formic acid liberated by the mannose and glucose required 8.21 ml. of 0.0106N sodium hydroxide and by the rhamnose 5.96 ml. Hence the apparent degree of hydrolysis was 92.6%; corrected for the ash content the degree of hydrolysis was 94%.

Attempted determination of the Mannose:Glucose ratio in the polysaccharide

Several attempts were made to determine the mannose: glucose ratio in the polysaccharide by eluting the mannose and glucose separately from a paper chromatogram and estimating them by periodate oxidation in the manner

described above. The results differed widely because of the difficulty of separating these two sugars completely. In four different determinations the following results were obtained, 2.85:1, 1.90:1, 2.54:1, and 2.10:1. These results suggest a value of 2-2.5:1, in agreement with visual estimation.

Periodate oxidation of Spruce glucomannan

Determination of periodate uptake.

Samples of the polysaccharide (0.30 mg.) were shaken in the dark with 0.3M sodium metaperiodate solution (12 ml.). The glucomannan dissolved in a few hours. At intervals a bottle was removed and a 5 ml. sample withdrawn. The solution was diluted to 50 ml., sodium bicarbonate (1g.) was added followed by a known excess of 0.1N sodium arsenite followed by potassium iodide (1g.) After standing for 15 minutes the excess arsenite was titrated with iodine solution (0.03602N).

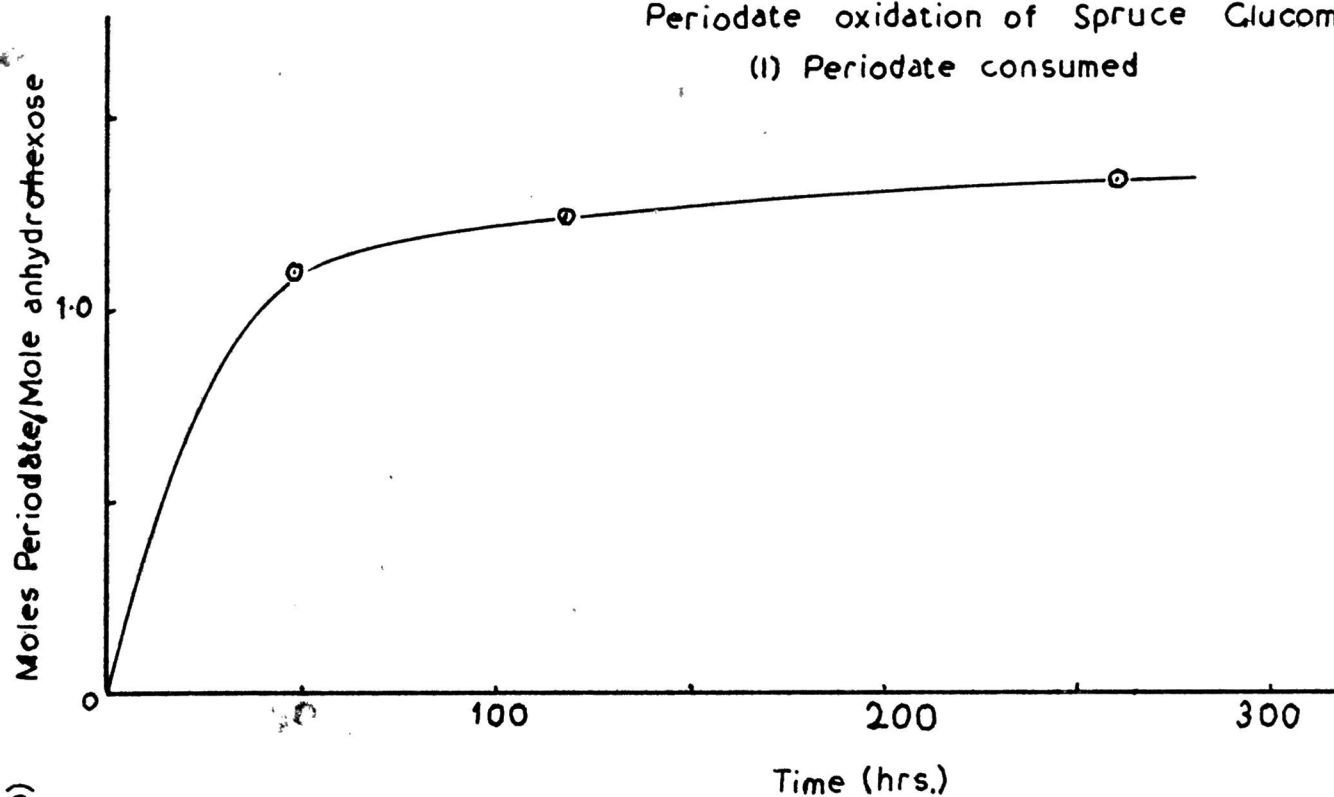
A reagent blank titration was also carried out. The difference in the titres was equivalent to the periodate consumed.

Results

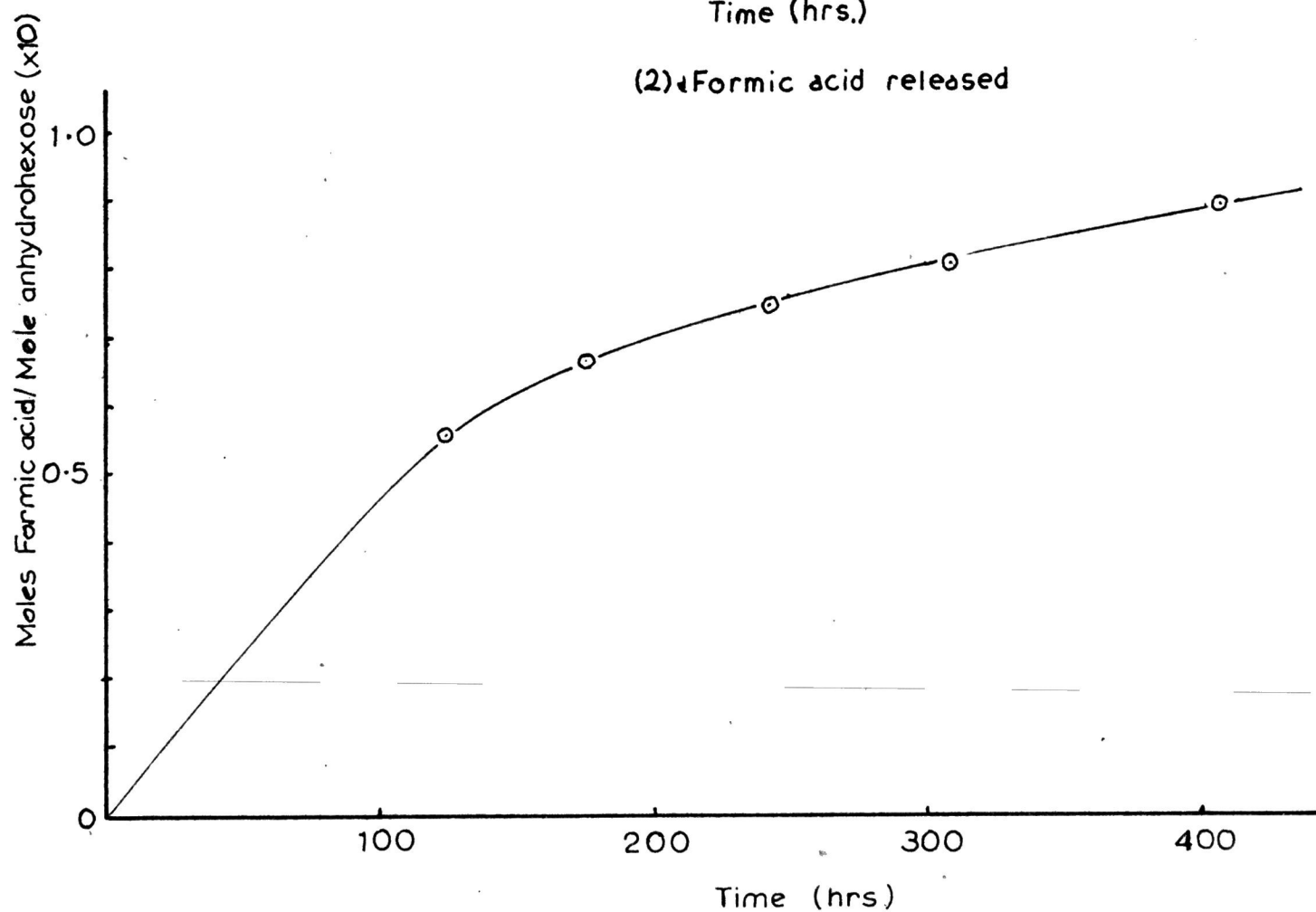
<u>Time (hrs.)</u>	<u>Wt. of poly-saccharide (mg.)</u>	<u>Titre (ml.)</u>	<u>Periodate uptake moles/mole anhydro-mannose</u>
48	28.25	4.50	1.11
117	35.13	6.28	1.24
261	35.00	6.71	1.34

GRAPH No. 3.

Periodate oxidation of Spruce Glucomannan
(1) Periodate consumed



(2) Formic acid released



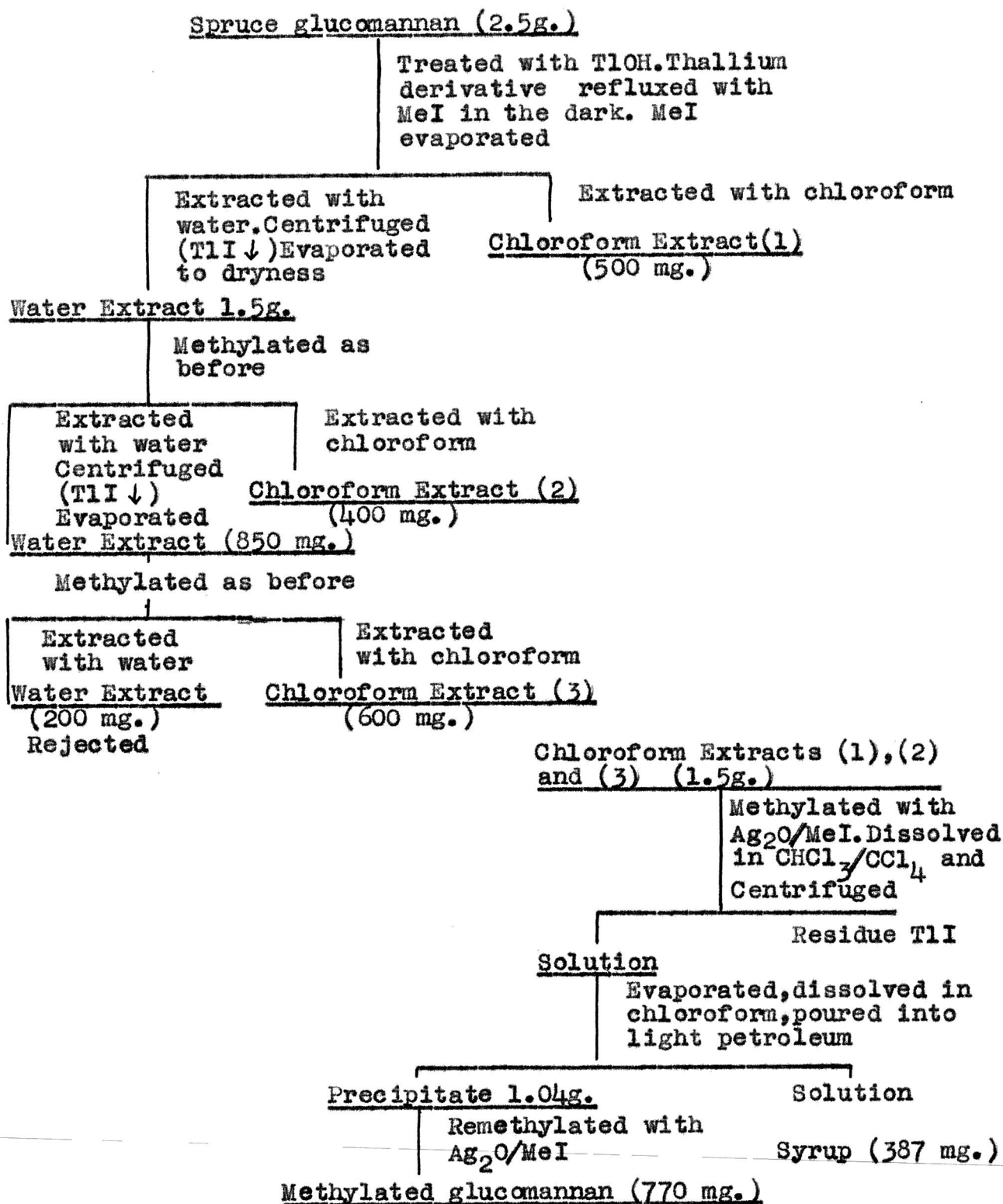
Determination of the formic acid released

The formic acid released was estimated by the method of Anderson, Greenwood, and Hirst (103). The polysaccharide (251.4 mg.) was treated with 0.56 M potassium chloride solution (45 ml.) and 0.2M sodium periodate solution (15 ml.). A blank, with reagents only, was also prepared. The mixture was shaken in the dark and at intervals samples (10 ml.) were withdrawn, treated with ethylene glycol (1 ml.) to destroy excess periodate and titrated to pH 5.8 with 0.0106 N sodium hydroxide solution, using a pH meter.

<u>Results</u>	<u>Time (hrs.)</u>	<u>Titre (ml.)</u>	<u>Moles anhydromannose/ mole formic acid</u>
	125	1.36	18.0
	177	1.62	15.1
	243	1.81	13.5
	310	1.94	12.4
	407	2.18	11.2

Methylation of Spruce glucomannan III

The glucomannan was methylated by the thallium method as described by Hirst and Jones (104). The scheme adopted is shown in the diagram on p.96 . The polysaccharide (2.5g.) was stirred in the dark with water (20 ml.) under a slow stream of nitrogen. Thallous hydroxide solution (10g. in 50 ml. water), prepared by adding barium hydroxide solution to a hot solution of thallous sulphate



(under soda-lime), was added slowly with stirring, and the mixture was stirred overnight to complete the reaction. The greyish-white precipitate was filtered off, washed with ethanol, and dried at 60°C/20 mm over phosphorus pentoxide. Later it was found best to freeze-dry at this stage since the dried thallium derivative is extremely hard. All these operations were conducted in the dark. Yield 6.9 g.

The product was powdered and treated with methyl iodide (30 ml.) and methanol (2 ml.) under reflux, in the absence of light and moisture, for 12 hours. The methyl iodide was distilled off and the residues extracted in a Soxhlet extractor with chloroform and methanol. The solvents were evaporated under reduced pressure, leaving a residue which was contaminated with colloidal thalious iodide.

Yield: c.500 mg.

The residues were shaken with cold water (200 ml.) and the mixture centrifuged at 5000 r.p.m. at 10°C. Evaporation under reduced pressure yielded a material which gave a positive Molisch test. A second extraction gave only a very small yield. The combined extracts were dissolved and centrifuged once more. This procedure removed most of the inorganic matter. Yield: c.1.5g.

Two further methylations were carried out in the same

way using the water-soluble fractions as the starting material. The remaining water-soluble material (200 mg.) was then rejected.

The chloroform-soluble fractions were combined and remethylated with methyl iodide (25 ml.) and silver oxide (3g.). After the removal of the methyl iodide, the residues were exhaustively extracted with chloroform. The solid left after evaporation of the chloroform was still contaminated with thallous iodide, but extraction with a 1:1 (v/v) mixture of chloroform and carbon tetrachloride followed by centrifugation at 5000 r.p.m. gave a thallium-free solution of the methylated polysaccharide. The product was purified by dissolving it in a small volume of chloroform and pouring into a large excess of light petroleum (B.P. 60-80°C). The white precipitate was separated at the centrifuge, washed with light petroleum, and freed of solvent under high vacuum. This material was considered to be methylated spruce glucomannan. Yield: 1.04g.

Ome : 41.8%

The glucomannan was remethylated with silver oxide and methyl iodide but the methoxyl content remained virtually constant at 42.0%. The methylated glucomannan had $[\alpha]_D^{19}$

- 13°(c, 2.48) in chloroform.

A sample (c. 5 mg.) of the methylated polysaccharide was hydrolysed by heating for 5 hours in anhydrous formic acid. Chromatographic examination of the hydrolysate (solvent, n-butanol-ethanol-water) showed that the main product had the same chromatographic mobility (R_G value, 0.84) as the 2:3:6-tri-O-methyl derivatives of D-mannose and D-glucose. In addition there were smaller amounts of two other components, one of which had the same chromatographic mobility (R_G , 1) as the 2:3:4:6-tetra-O-methyl derivatives of mannose and glucose, and the other an R_G value (0.37) corresponding to a di-O-methyl hexose.

The mother-liquor from the purification of the methylated glucomannan was evaporated under reduced pressure and gave a considerable amount (387 mg.) of a yellow syrup. On hydrolysis it yielded the same products as the methylated polysaccharide, but the proportions of the di-O-methyl and tetra-O-methyl hexoses appeared to be much higher particularly in the case of the latter. The fraction could not be obtained solid and because of purification difficulties was not examined further.

Hydrolysis of the methylated glucomannan

The methylated product (650 mg.) was dissolved in anhydrous formic acid (20 ml.) and heated for 5 hours at

100°C in a sealed tube. The solutions became very dark and some destruction of the material obviously occurred. The formic acid was removed and formyl esters hydrolysed in the usual way, and after neutralisation the solution was concentrated to a syrup and dried over phosphorus pentoxide. Yield: 536 mg.

Separation of the hydrolysis products on a cellulose column

A cellulose column 21 x 530 mm. was prepared and washed with water (200 ml.) and solvent (200 ml.). The solvent employed was methyl-ethyl-ketone, saturated with water. The methylated sugars were applied on 12 discs of 3MM paper and elution commenced using a constant-level reservoir. Fractions (4-6 ml.) were collected in small test-tubes on an automatic turntable. The elution was followed in the usual way by analysing the contents of every tenth tube chromatographically. Four main fractions were obtained. The recovery was 92%.

Fraction 1 (15 mg.) Tubes 26-39. This fraction was obtained as a syrup and had $[\alpha]_D^{19} +8^\circ$. It gave a pink spot with aniline oxalate and had the same chromatographic mobility in n-butanol-ethanol-water ($R_F, 1.0$) and in benzene-ethanol-water, as the 2:3:4:6 - tetra-O-methyl derivatives of D-mannose and D-glucose. There was no indication, on the

chromatogram run in the latter solvent, of any trace of 2:3:4:6-tetra-O-methyl-D-galactose.

Fraction 2 (367 mg.) Tubes 40-80. This fraction gave a single spot on a paper chromatogram which had the same R_F value (0.84) as the 2:3:6-tri-O-methyl derivatives of D-mannose and D-glucose. The syrup had $[\alpha]_D + 10.2^\circ$, (c , 3.5 in water) suggesting that it was a mixture of these sugars.

Fraction 3 (92 mg.) Tubes 85-140. This fraction appeared to be a mixture of di-O-methyl hexose with a pentose derivative which gave a pink spot of virtually the same R_F value (0.55).

Fraction 4 (20 mg.) Tubes 140-170 and water wash (150 ml.) This fraction had a chromatographic mobility corresponding to a mono-O-methyl hexose (R_F , 0.37).

Separation of the tri-O-methyl hexoses by selective furanoside formation

Rebers and Smith (22) have separated 2:3:6-tri-O-methyl-D-mannose and 2:3:6-tri-O-methyl-D-glucose by treatment with cold dilute methanolic hydrogen chloride. Methyl-2:3:6-tri-O-methyl glucofuranoside is formed preferentially and may be separated from the reducing sugar by virtue of its greater chromatographic mobility.

A portion of Fraction 2 (67 mg.) was dissolved in 1% methanolic hydrogen chloride and allowed to stand at room

temperature. The solution had $[\alpha]_D^{17} +22^\circ$ (initial value) falling to -4° (constant value) after 20 hours.

After neutralisation with silver carbonate the solution was concentrated to a syrup. The syrup was dissolved in methyl-ethyl-ketone (5 ml.) and absorbed onto a cellulose column $1\frac{1}{4} \times 400$ mm. The column was eluted with methyl-ethyl-ketone (water-saturated) and fractions (2-4 ml.) were collected in the usual way on a turntable. Every fifth tube was evaporated to dryness in a current of air and the contents tested by spotting onto filter-paper and spraying and heating with aniline oxalate solution. In this way a non-reducing and a reducing fraction were obtained.

Fraction 1 (tubes 1 - 25) 23 mg. Non-reducing.

Fraction 2 (tubes 30-55) 41 mg. Reducing.

Fraction 1 The syrup was hydrolysed by heating for 2 hours at 100°C in 0.1N sulphuric acid (1 ml.). The solution was neutralised and evaporated to give a syrup (19 mg.) which was strongly reducing. The syrup was dissolved in dry pyridine and treated with p.mitrobenzoyl chloride (85 mg.) at $65-75^\circ\text{C}$ for 30 minutes. The solution was allowed to stand overnight and saturated sodium bicarbonate solution added dropwise until no further

effervescence occurred. Water (3 ml.) was added and the solution extracted three times with chloroform (10 ml.). The extract was dried over anhydrous sodium sulphate and the chloroform evaporated under reduced pressure. The crystalline residue was re-crystallised from methanol. After two recrystallisations it melted at 187-189.5°C. undepressed on admixture with an authentic specimen of the di-p-nitrobenzoate of 2:3:6-tri-O-methyl-D-glucose. The authentic sample melted at 189-190°C. which is the same value as that reported by Rebers and Smith (22).

Fraction 2 The di-p-nitrobenzoyl ester was prepared in the manner described above. After several recrystallisations the product melted at 181.5-185°C. undepressed on admixture with an authentic sample of the di-p-nitrobenzoyl ester of 2:3:6-tri-O-methyl-D-mannose. The authentic preparation melted at 187-188°C., the value reported by Rebers and Smith (loc. cit.). A mixture of the authentic di-p-nitrobenzoates of 2:3:6-tri-O-methyl-D-mannose and 2:3:6-tri-O-methyl-D-glucose melted at 167-173°C.

Estimation of the ratio of tri-O-methyl-D-mannose to tri-O-methyl-D-glucose

1. The mixture had $[\alpha]_D^{25} +10.8^\circ$ (c, 3.47 in water).

The specific rotation of 2:3:6-tri-O-methyl-D-glucose is $+70.5^{\circ}$. Taking the specific rotation of 2:3:6-tri-O-methyl-D-mannose as -10° the ratio of the two sugars is 26:74.

2. The optical rotation of the mixture in dilute methanolic hydrogen chloride changed from $+22^{\circ}$ to -4° . Since the specific rotation of 2:3:6-tri-O-methyl-D-glucose changes from $+70^{\circ}$ to -33° in this solvent while that of 2:3:6-tri-O-methyl-D-mannose is unchanged the proportion of the tri-O-methyl-D-glucose in the mixture is 25%.

Acetolysis of the glucomannan

Small-scale experiments on the acetolysis of the glucomannan showed that a reasonable yield of oligosaccharide could be obtained using a reaction time of 120 hours. Accordingly, a sample (1.0g.) of the glucomannan was treated for this period with a mixture of acetic anhydride (12 ml.) glacial acetic acid (12 ml.) and concentrated sulphuric acid (1.2 ml.). Although not all the material appeared to have dissolved the mixture was poured into ice-water and the sugar acetates extracted with chloroform. The extract was dried over anhydrous sodium sulphate and the solvent evaporated under reduced

pressure.

Yield: 1.0 g.

The acetates were dissolved as far as possible in absolute methanol (25 ml.) and saponified by treatment with N barium methoxide solution (1 ml.). The equivalent volume of dilute sulphuric acid was added followed by water (25 ml.), and the solution filtered through sintered glass and concentrated to a syrup.

Yield: 360 mg.

The products were examined on a paper chromatogram in ethyl acetate-pyridine-water. The following monosaccharides were detected: mannose (principally), glucose, galactose, and xylose. Two other components were also clearly distinguished, the more prominent (A) had the same chromatographic mobility as mannobiose and cellobiose (R_M 0.52), the other (B) travelled at the same rate as the mannosyl glucose isolated from the ivory nut mannose^{an.} (R_M 0.38). Traces of two other components, presumably disaccharides, having mobilities intermediate between those of galactose and mannobiose, were also detected.

A portion (c.150 mg.) of the syrup was separated on strips of Whatman 3 MM paper. The portions containing components A and B (as revealed by spraying side-strips which were cut off and eluted with cold water).

Component A (c.20 mg.) gave a single spot on a paper chromatogram but when analysed by paper ionophoresis (in borate buffer, pH 10 at a p.d. of 700v) it was shown to consist of a mixture of two sugars having the same ionophoretic mobilities as mannobiose and cellobiose. On hydrolysis (3 hours N sulphuric acid) the mixture gave mannose and glucose.

Component B (c.8 mg.) This component was also analysed by paper ionophoresis and appeared to consist of a single sugar having the same ionophoretic mobility as mannosyl glucose. The syrup had $[\alpha]_D^{20} +9^{\circ}$ (c,2.3 in water). On hydrolysis (3 hours N sulphuric acid) it gave mannose and glucose in equal proportions. On borohydride reduction and hydrolysis it gave only mannose.

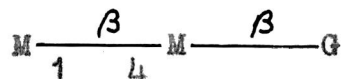
SUMMARY

The Mannans of Ivory Nut

Mannan A

1. Mannan A has been extracted from delignified ivory nut shavings with 7% potassium hydroxide and precipitated from the neutralised solution with methylated spirits. The polysaccharide appears to be identical with those obtained by earlier workers.
2. Mannan A was subjected to a graded acetolysis by treatment at room temperature with a mixture of acetic anhydride, acetic acid, and concentrated sulphuric acid.
3. The resulting mixture of oligosaccharide acetates was deacetylated with barium methoxide and the free sugars fractionated on a charcoal-celite column. The following sugars were isolated and crystallised: (β -1:4)mannobiose, mannotriose, mannotetraose, and mannopentaose. The latter two have not been fully characterised.
4. Fractions containing mixtures of di- and trisaccharides were separated on cellulose. Chromatographically pure samples of two disaccharides and two trisaccharides have been obtained. The disaccharides were shown to be α -1:4 mannobiose, and a β -linked mannosyl glucose. The latter

is linked either in the 3 or the 4-position. Isomannotriose, which has an α -1:4 link at the non-reducing end and a β -1:4 link at the reducing end, has been crystallised. The other trisaccharide has the structure:



5. Two other fractions were obtained having as their principal constituents a tetraose and a pentaose which appear to be homologues of isomannobiose and isomannotriose.
6. The structure of Mannan A has been discussed in the light of these results and of the results of earlier methylation studies.

Mannan B

1. Mannan B has been extracted with a cuprammonium hydroxide solution from the residues of the Mannan A extraction and precipitated as the copper complex with sodium hydroxide. The complex was decomposed with acetic acid and extracted with dilute alkali to remove Mannan A. Mannan B was extracted with anhydrous formic acid from the residues. The mannan was shown to be closely similar to the products obtained by earlier workers.

2. Mannan B was subjected to a graded acetolysis. The product was saponified and the free sugars fractionated on charcoal.
3. Mannobiose, mannotriose, and mannotetraose were isolated in a pure condition, and crystallised.
4. Isomannobiose, isomannotriose, mannosyl glucose, and glucomannotriose have also been isolated.
5. These results, together with the earlier methylation studies, suggest that the chief difference between Mannan A and Mannan B is one of molecular size.

Sitka Spruce Glucomannan

1. A glucomannan isolated from Sitka Spruce by Dr. R.A.Laidlaw has been the subject of a preliminary investigation.
2. The glucomannan was oxidised with the periodate ion. The uptake of periodate was 1.14 moles/mole of anhydrohexose, suggesting that the polysaccharide was either 1:4 or, less probably, 1:2-linked.
3. The glucomannan was methylated, initially with thallous hydroxide and methyl iodide and afterwards with silver oxide and methyl iodide. Methylation was incomplete.

4. The methylated product was hydrolysed and the products separated on cellulose. The principal fraction obtained was shown to be a mixture of 2:3:6-tri-O-methyl-D-glucose and 2:3:6-tri-O-methyl-D-mannose, in the ratio 1:3. The end-group fraction has been tentatively identified as 2:3:4:6-tetra-O-methyl-D-mannose. No tetra-O-methyl-D-galactose was present.
 5. The glucomannan was subjected to a graded acetolysis. The products were deacetylated and a portion separated chromatographically on thick paper. The main disaccharide fraction has been shown to be a mixture of mannobiose and cellobiose. A third disaccharide appeared to be identical with the mannosyl glucose obtained from the ivory nut mannans.
 6. The structural significance of these results has been discussed.
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